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Elevated expression of members of the epidermal growth factor receptor (EGFR) family has been frequently observed in a large number of sporadic breast cancers. For example, overexpression and amplification of the Neu/ErbB-2 protooncogene has been implicated as an important determinant in the initiation and progression of human breast cancer. To directly test the importance of the Neu in mammary tumor progression, we have derived transgenic mice that express Neu under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer. The induction of mammary tumors in these strains was further correlated with elevated expression of ErbB-3 EGFR family member. To explore whether the catalytic activity of the EGFR is required for the induction of mammary tumors in these Neu strains, we have crossed the Neu transgenic mice with the Waved-2 strain which encode a catalytically inactive EGFR. The results of this cross revealed that the catalytic activity of EGFR is dispensable for Neu tumorigenesis. Taken together these observations argue that ErbB-3 and Neu are the principle EGFR family members involved in the induction of mammary carcinoma. We are currently testing the importance of erbB-3 in mammary tumorigenesis by interbreeding MMTV/erbB-3 strains with the MMTV/Neu strains.

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Introduction

The progression of a primary mammary epithelial cell to the malignant phenotype is thought to involve multiple genetic events including the activation of dominant acting oncogenes and the loss of specific tumor suppressor genes. Activation of certain tyrosine kinases have been implicated in the malignant progression of a significant proportion of human breast cancers. Oncogene products which either possess constitutively-activated tyrosine kinase activities are particularly potent in their capacity to transform the mammary epithelium of transgenic mice. Of particular relevance to this proposal is the observation that members of the Epidermal growth factor receptor tyrosine kinases (EGFR) family have been implicated in human breast cancer. The EGFR family comprises four closely related type 1 receptor tyrosine kinases (RTKs) (EGFR, Neu{erbB-2,HER2}, erbB-3, and erbB-4) (Ullrich and Schlessinger, 1990). Elevated levels of expression of the various EGFR family members have been observed in both primary breast cancers and their derived cell lines. For example, amplification and consequent overexpression of Neu has been observed in a significant proportion of primary human breast cancers (King et al. 1985; Slamon et al., 1987 ; Slamon et al., 1989). Moreover, the extent of overexpression of Neu has been also inversely correlated with patient survival (Patterson et al., 1991; Gullick, et al. 1991). More recent studies have implicated the expression other EGFR family members including EGFR, erbB-3 and erbB-4 in the genesis of human breast cancer (Kraus et al., 1993; Plowman et al., 1993)

These observations suggest that the expression of the different members of the EGFR family members and their cognate ligands may play a crucial role in mammary tumorigenesis. In addition to these transgenic studies, there is a large body of evidence implicating the expression of the various EGFR family ligands and their cognate ligands in the induction of mammary tumors. For example, the expression of EGFR specific ligands such as TGFα (transforming growth factor) and EGF (epidermal growth factor) can be detected in primary breast cancers (Salomon et al., 1990). Moreover expression of several members of erbB-3 and erbB-4 ligands collectively known as the NDFs (Neu differentiation factors) or heregulin have also been implicated in the pathogenesis of human breast cancer (Holmes et al., 1992, Wen et al., 1992; Krane and Leder, 1996). Although Neu cannot bind either the NDF or EGF ligands, its activity can be profoundly influenced by the expression of these growth factors. For example, Neu is the substrate of the activated EGFR following stimulation of cells with EGF or TGFα (Stern and Kamps, 1988, Kokai et al., 1988, Goldman et al., 1990). Similarly, Neu can be transphosphorylated by either erbB-3 and erbB-4 ligands following stimulation of mammary tumor cells with NDFs (Karaunagan et al., 1996). The ability of these growth factors to modulate the activity of Neu is thought to be mediated through the formation of specific heterodimers of Neu and the different EGFR family members (Wada et al., 1990; Karaunagan et al., 1996, Tzhar et al., 1996, Pinkas-Kramarski et al., 1996; Graus-Porta et al., 1997). Consistent with these observations, co-expression of Neu and EGFR or Neu and erbB-3 results in the efficient transformation of fibroblasts in vitro (Kokai et al. 1989, Alimandi et al., 1995).

The central importance of Neu in this hierarchy of EGFR receptor interactions has been recently been demonstrated by ablating Neu function in breast cancer cells by use of a single chain Neu specific antibodies. Induction of the single chain antibody in these cells ablates the ability of these breast cancer cell lines to respond to the mitogenic stimulation of both EGFs and NDFs (Graus-Porta et al., 1995). Moreover these investigators further demonstrated that the erbB-2 is the

preferred heterodimerization partner for EGFR or erb-B-3/erbB-4 following stimulation with either EGFs or NDFs respectively (Graus-Porta et al., 1997). The molecular basis for the cooperative ability of the different EGFR family may reflect the distinct signaling specificity of the different heterodimers. For example, activation of the erbB-3 by the NDFs is thought to recruit the phosphatidylinositol 3' (PI-3') kinase to Neu through the formation of Neu/erbB-3 heterodimers (Pringent and Gullick 1994; Soltoff et al., 1994). Indeed, the erbB-3 RTK possesses 6 consensus tyrosine autophosphorylation binding sites for the SH2 domain of the p85 subunit of the PI-3' kinase. Conversely, Neu appears to the principle binding partner for the Src family of tyrosine kinases (Muthuswamy et al. 1994; Muthuswamy and Muller 1995).

Direct evidence for the importance of these EGFR family members and their cognate ligands in mammary tumors derives from studies of transgenic mice that have been engineered to over express these genes in the mammary epithelium. For example, mammary epithelial-specific expression of activated form of the *neu* oncogene, which is associated with constitutive tyrosine kinase activity, leads to the rapid development of multifocal mammary tumors in every female transgene carrier examined (Muller et al., 1988; Guy et al., 1996; Bouchard et al. 1989). Consistent with these observations, mammary-epithelial expression of the *neu* proto-oncogene results in the induction of focal mammary tumors which frequently metastasize to the lung (Guy et al., 1992). Although the levels of Neu protein in normal and tumor tissue appear to be equivalent, biochemical analyses of these tumors has revealed that tumor epithelia possesses elevated levels of tyrosine phosphorylated Neu compared to the adjacent normal epithelium (Guy et al., 1992). One possible explanation for these observations is that tumor progression in these strains may involve acquisition of activating mutations in the *neu* transgene. Indeed several studies have demonstrated that mutations in either the extracellular transmembrane or carboxyl-terminal regulatory domain of Neu can lead to its oncogenic activation (Bargmann et al, 1986; Bargmann and Weinberg, 1988). Because activation of Neu can occur through the mutation of a single amino acid in the transmembrane domain (Bargmann et al., 1986), we investigated whether this mutation could be detected during tumor progression in these strains. The results of these analyses revealed that over 70% of the primary mammary tumors arising in these strains contained in frame deletions in the transgene (Siegel et al., 1994). Sequence analyses of the altered regions revealed that it resides in a cysteine rich region which is highly conserved in members of the EGFR family. Significantly, these sequence alterations appear to affect the balance of cysteine residues within this region by either deleting or inserting single cysteine residues (Siegel and Muller, 1996). More recently, we have demonstrated that in addition to insertions and deletions, the balance of cysteine residues can be affected by single point mutations which result in the either conversion a particular amino acid to cysteine or alteration of an existing cysteine residue to another amino acid (Siegel and Muller, 1996). As a result of this cysteine imbalance the mutated Neu receptors dimerize through cysteine disulfide bridges leading to constitutive activation (Siegel and Muller, 1996).

Given the importance of the EGFR family in mammary tumorigeneseis the primary focus of our Army sponsored research program has been to assess the relative contribution of the individual EGFR family members to mammary tumorigenesis and metastases. Our initial objective was designed to evaluate the importance of the EGFR in Neu-induced tumorigenesis. In one set of experiments, we and our collaborators have examined whether coexpression of EGFR ligand, TGFα and Neu in the mammary epithelium could act cooperatively to transform the mammary epithelial cell. The results revealed that coexpression of TGFα and Neu resulted in a dramatic acceleration of mammary tumors (Muller et al., 1996). Another important set of experiments attempted to address

whether the activity of the EGFR was necessary for Neu-induced mammary tumors. To accomplish this we interbred the MMTV(mouse mammary tumor virus)/*neu* mice with a naturally occurring mouse mutant that possessed a kinase dead EGFR (Waved-2 mouse) (Lutteke et al., 1994; Fowler et al., 1995). Although the rate of tumors was dramatically decreased in genetic background carrying the Waved-2 mutation, the rate of tumors was identical between wild type and mice homozygous for the Waved-2 mutation. Taken together these observations suggest that the catalytic activity of the EGFR is dispensable for Neu mediated tumorigenesis.

In other series of experiments we identified the region of Neu that is involved in the differential recruitment of c-Src. Because previous studies have suggested that Neu is the preferential binding partner for c-Src in a EGFR/Neu heterodimer (Muthuswamy and Muller, 1995) , we have used EGFR/Neu chimeric receptors to map the c-Src binding site to the carboxyl region of the catalytic domain of Neu. We have further mapped the binding site to one of two tyrosine phosphorylation sites within this region by creating a series of phenylalanine site specific mutants. The final specific objective of our Army sponsored research program was to assess the relative importance of the erbB-3 and erbB-4 EGFR family members in mammary tumorigenesis. Examination of the levels of the various EGFR family members in mammary tumors derived from transgenic mice expressing elevated activated *neu* revealed that the amongst the EGFR family members, erbB-3 is expressed at elevated levels compared to the adjacent morphological epithelium. Interestingly, the observed upregulation of erbB-3 in Neu-induced tumorigenesis appears to occurring at either translation or posttranslational level since erbB-3 transcript levels are equivalent in both normal and tumor tissue. These observations suggest that Neu and erbB-3 levels are coordinately upregulated during mammary tumorigenesis. More recently, we have derived transgenic mice that express elevated levels of erbB-3 in the mammary epithelium. We are currently interbreeding the MMTV/erbB-3 strains with the activated *neu* strains to directly assess the importance of erbB-3 in mammary tumorigenesis.

Body

Synergistic interaction of the neu proto-oncogene and TGFα in mammary tumorigenesis.

To determine whether TGFα and Neu could cooperate in mammary tumorigenesis transgenic mice bearing the MMTV/TGFα and MMTV/neu proto-oncogene were interbred to generate F1 mice that carried either *neu*, TGFα or both transgenes (see Muller et al., 1996; appended manuscript). Because the TGFα females were unable to nurse their young, the bigenic strains were generated by crossing the MMTV/TGFα males with the MMTV/*neu* females. The MMTV/TGFα mice were derived from the line 29 strain (Matsui et al., 1990) whereas the MMTV/neu mice were derived from the N#202 lineage (Guy et al., 1992). To ascertain whether coexpression of TGFα and Neu resulted in an increase in the incidence of mammary tumors, virgin females from these crosses were monitored for the development of mammary tumors by physical palpation. The results of these analyses revealed bigenic animals carrying both TGFα and *neu* transgenes developed mammary tumors with accelerated kinetics relative to either parental strain (see Figure 3 Muller et al., 1996 appended manuscript). For example, whereas 50% of the bigenic female carriers had developed mammary tumors by 175 days of age, the female carriers carrying either TGFα or Neu alone had not yet developed mammary tumors. (See Figure 3, Muller et al. 1996). Another important

distinction between the bigenic female and single transgene carriers was revealed by wholemount analyses of the mammary epithelium. For example, the alveoli of the Neu/TGFα bigenic animals possessed a denser cell lining compared to the cystically dilated alveoli found in the TGFα mice (see Figure 4, Muller et al., 1996). Consistent with this whole mount analyses, histological examination of the mammary epithelium from the either monogenic or bigenic progeny revealed that only the latter could mammary epithelial dysplasias be detected (see Figure 5, Muller et al., 1996) Taken together, these observations suggest that coexpression of Neu and TGFα can act synergistically to transform the mammary epithelium.

To confirm that the phenotypes observed in the dual transgene carriers was a result of coexpression of Neu and TGFα transgenes, RNase protection analyses with probes specific for TGFα and *neu* (Melton et al., 1984; Matsui et al., 1990; Siegel et al. 1994) were conducted on 20 ug of total RNA derived from the mammary tissue samples of the various transgene carriers. Examination of RNA samples derived from tumors of eight MMTV/*neu* female mice revealed abundant *neu* transcripts (Figure 1, Muller et al. 1996). Interestingly, several of these tumor samples demonstrated evidence of altered *neu* transcripts (Figure 1, Muller et al., 1996). Indeed previous studies have demonstrated that these altered transcripts encode in-frame deletions in the extracellular domain which result in its oncogenic activation (Siegel et al., 1994, Siegel and Muller, 1996). Significantly, the mammary tumor samples obtained from seven dual transgene carriers did not exhibit evidence of altered transcripts that were observed in the Neu-induced tumors. (Figure 1A, Muller et al. 1996). Because these tumors possessed elevated levels of tyrosine phosphorylated Neu (Figure 7D, Muller et al., 1996), activation of Neu is likely occurring through transactivation of the Neu receptor.

An identical RNase protection analyses was performed on these RNA samples to assess the levels of TGFα (Figure 1B, see Muller et al., 1996). These analyses revealed that TGFα could be detected mammary tumor samples derived from both MMTV/TGFα mice (see Figure 2B , Muller et al., 1996) and mice carrying both *neu* and TGFα transgenes (see Figure 1B, Muller et al., 1996). Together with the histological observations, these findings suggest that coexpression of Neu and TGFα is associated with the rapid induction of mammary tumors.

To explore whether coexpression of TGFα and Neu in the mammary epithelium resulted in the concerted activation of Neu and EGFR, protein lysates from these tissues were immunoprecipitated with either Neu or EGFR specific antisera and immunoblotted with phosphotyrosine specific antibodies.The results of these analyses revealed that elevated levels of tyrosine phosphorylated Neu could be detected in tumors derived from either *neu* or *neu*/TGFα animals. By contrast, the TGFα induced tumors failed to display evidence for tyrosine phosphorylated Neu (Figure 7, Muller et al., 1996). However, a similar analyses for EGFR revealed low to variable levels of tyrosine phosphorylated EGFR in the various tumors (Figure 7, Muller et al., 1996). To explore the possibility that activation of Neu in *neu*/TGFα tumors occurs through the formation of specific heterodimers, tumor extracts from the *neu*/TGFα animals were subjected to reciprocal immunoprecipitation and immunoblot analyses with antisera specific to EGFR and Neu (Figure 8, Muller et al., 1996). Although elevated levels of EGFR and Neu could be detected in this particular subset of mammary tumors, we could not detect coimmunoprecipitation of Neu and EGFR under these experimental conditions. These findings indicate that the observed transactivation of Neu by EGFR does not involve the formation of stable Neu/EGFR heterodimers.

One possible explanation for the observed synergy between Neu and TGFα /EGFR is that these closely related type 1 receptors recruit distinct but complimentary signaling molecules. Indeed,

we have previously demonstrated that activation of c-Src by EGFR in fibroblasts is mediated by the direct and specific interaction of c-Src with Neu (Muthuswamy and Muller, 1995). To confirm whether c-Src was recruited to Neu in the Neu /TGFα tumors we first examined whether a radiolabeled Src SH2 probe could interact directly with Neu in the Neu/TGFα tumors. The results of these analyses revealed that Src SH2 domain could directly interact with Neu in these tumors (Figure 9A, Muller et al., 1996). Consistent with this far western analyses coimmunoprecipitation analyses with Neu and c-Src specific antisera demonstrated that specific complexes of c-Src and Neu could be detected in the TGFα /Neu tumors (Figure 9B, Muller et al., 1996). Taken together, these observations argue that transactivation of Neu by TGFα results in the recruitment of the c-Src signaling pathway.

The differential interaction of the Src family kinases with the EGFR and Neu resides in the catalytic domain of Neu.

Whereas activation of Src by TGFα/EGFR is mediated through its specific and direct interaction of Neu, the site on Neu responsible for this interaction has not yet been identified. Given that the interaction of c-Src with Neu occurs through the interaction of the c-Src SH2 domain and a specific phosphotyrosine residue located on Neu (Muthuswamy and Muller, 1995), we initially examined whether an activated Neu molecule lacking the 5 known tyrosine phosphorylation sites was capable of interacting with Neu. This mutant known as NT-NYPD contains phenylalanine substitutions at each of the known tyrosine phosphorylation sites in the context of an oncogenic activating mutation in the transmembrane domain of Neu (Figure 1C, Appendix 1, Dankort et al., 1997). Analyses of Neu immunoprecipitates from this mutant using a glutathionine-S-transferase c-Src SH2 domains revealed that the NT-NYPD mutant (or mutants harboring single tyrosine phosphorylation sites, NT-YA-YE) still retained the capacity bind a radiolabelled c-Src probe (Figure 2A, lanes 8-13, Appendix 1). Consistent with these far western analyses, coimmunoprecipitation analyses with Src and Neu specific antisera revealed that the NT-NYPD mutant retained its capacity to associate with c-Src (Figure 2C, lane 3, Appendix 1). These findings suggest that the major Neu autophosphorylation sites are dispensable for c-Src binding.

To further localize the site in Neu responsible for the differential interaction of Src family kinases, chimeric receptors comprising different segments of the EGFR and Neu were obtained from the laboratory of Dr. P. DiFiore (DiFiore et al., 1990). As shown in Figure 3 (Appendix1), all the chimeric receptors possess the EGFR ligand binding domain and the carboxyl region harboring the EGFR major tyrosine autophosphorylation sites. However, the various chimeric receptors possess different segments of the Neu catalytic and transmembrane domains (Figure 3, Appendix 1). In order to assess whether the catalytic domain of Neu was responsible for mediating interaction of Neu with Src family kinases stable Rat-1 cell lines expressing these chimeric receptors were derived (Figure 3, Appendix 1: WT, TMTK, TK1, RT, RHER). To confirm that these cell lines were expressing the various chimeric receptors, protein lysates derived from these lines were subjected to immunoblot analyses with EGFR specific antisera. The results revealed that several selected cell lines expressed elevated levels of the various chimeric receptors (Figure 4A, Appendix 1).

Because the chimeric receptors harbor the EGFR ligand binding domain their activity can be stimulated by addition of the EGF ligand. As shown in Figure 4B(Appendix 1), addition of EGF to these various cell lines resulted in a marked stimulation of the tyrosine kinase activity the chimeric receptors. Interestingly two of the cell lines expressing the chimeric receptors possessed high levels

of tyrosine phosphorylated protein even in the absence of EGF stimulation (TMTK, TK Figure 4B, lanes 3, 5, Appendix 1). To assess whether these different chimeric receptors were capable of associating with c-Src, protein lysates from these cell lines were subjected immunoprecipitation/ immunoblot analyses with Src and EGFR specific antisera. The results of these analyses revealed that both the TMTK and TK cell lines were capable of binding c-Src under both unstimulated and stimulated conditions (Figure 4C, lanes 3-6, Appendix 1). In contrast to these observations, the chimeric receptors derived from TK1 and RT receptors and the wild type EGFR failed to associate with c-Src (Figure 4C, lanes 1-2, 7-12, Appendix 1). The inability of these receptors to bind c-Src was not due to differing levels of c-Src since these cell lines expressed equivalent levels of c-Src (Figure 4D, Appendix 1). Taken together, these observations suggest that the c-Src binding site resides in the Neu catalytic domain. Since the TK1 chimeric receptor failed to bind c-Src these data further argue that the c-Src binding site resides in the carboxyl region of the catalytic domain (TK2) (Figure 3, Appendix 1).

To confirm that the TK2 region was sufficient to confer c-Src binding, we constructed a chimeric EGFR receptor bearing the TK2 region of Neu and stable Rat-1 cell lines expressing the TK2 chimeric receptor were derived. To confirm that these cell lines expressed biologically active EGFR, cell lines expressing the TK, TK2 and wild type EGFR were stimulated with EGF and the cell lysates were subjected to immunoprecipitation/immunoblot analyses with EGFR and phosphotyrosine specific antibodies. Although tyrosine phosphorylated EGFR was detected upon EGF stimulation in all cell lines (Figure 5A, lanes 2,4, 6, Appendix 1) both the TK and TK2 chimeric receptors displayed elevated basal levels of tyrosine phosphorylated EGFR in the absence of EGF. To determine if the TK2 chimeric receptor was capable of binding c-Src the protein lysates were subjected to immunoprecipitation/immunoblot analyses with Src and EGFR specific antisera (Figure 5B, Appendix 1). The results showed that both the TK and TK2 chimeric receptors were capable of binding c-Src whereas the wild type EGFR failed to complex with c-Src (Figure 5B, Appendix 1). Again the inability to detect c-Src coimmunoprecipitation in these lines was not due to differences in the levels of c-Src since all cell lines expressed equivalent levels of c-Src (Figure 5C, Appendix 1). Taken together, these findings argue that c-Src binding site is localized to the TK2 region of Neu.

Because c-Src binding is dependent on specific phosphotyrosine residues, four potential phosphotyrosine residues within the TK2 region represent candidate binding sites for c-Src. To further define the c-Src binding site we created single phenylalanine substitutions within the four tyrosine residues located in the TK2 (Figure 6A, YHAD-F, YGVT-F, YDGI-F, YMIM-F, Appendix 1) region in activated form of Neu. As an initial test of the biological activity of these various mutations we measured the specific transforming activity of these various mutations in Rat-fibroblasts. The results of these analyses revealed that mutation of two of the sites resulted in a dramatic decrease in the specific transforming activity (Figure 6B, YHAD-F, YGFT-F, Appendix 1) whereas mutation of remaining two sites resulted in enhanced transforming activity (Figure 6B, YDGI-F, YMIM-F, Appendix 1). To determine if the reduced transforming activity in the former set of mutants reflected impairment of the catalytic activity we established stable cell lines expressing these mutant Neu receptors and measured the state of tyrosine phosphorylation of Neu in these cells (Figure 7A, Appendix 1). The results of these analyses revealed that the two transformation competent Neu mutants were tyrosine phosphorylated whereas the defective transforming mutants were catalytically inert (Figure 7A, Appendix 1). We further demonstrated that the two transforming mutants retained their ability to associate with c-Src (Figure 7B, Appendix 1).

Because of the low levels of tyrosine phosphorylated nontransforming mutant receptors, it is difficult to ascertain which of the two remaining two tyrosine phosphorylation sites is the authentic binding site remains to be assessed. However, the precise identification of the c-Src binding site on Neu awaits further molecular analyses (see Conclusions).

The catalytic activity of the EGFR is dispensable for Neu-mediated mammary tumorigenesis.

Although our data suggest that TGFα and Neu can act cooperatively to transform the mammary epithelium, whether the catalytic activity of the EGFR is required for the induction of mammary tumors is unclear. To assess the role of the various EGFR members in mammary tumorigenesis, we have derived transgenic mice which express a constitutively activated versions of Neu in the mammary glands (NDL [Neu deletion] strains). Unlike the parental MMTV/wild type neu strains which developed focal mammary tumors with a variable onset, the NDL strains developed multifocal mammary tumors with in a relatively synchronous fashion (Figure 8, Appendix 1). The rapid onset of tumors in these strains was further correlated with high with high levels of expression of the NDL transgene (Figure 9, Appendix 1) and elevated levels of tyrosine phosphorylated Neu (Figure 10, Appendix 1). To assess whether the catalytic activity of the EGFR is required for the induction of mammary tumors by Neu, we have interbred one of these NDL strains (NDL1-2) to a naturally occurring mouse mutant which encodes a catalytically impaired EGFR (Waved-2 mouse, Luetteke et al., 1994; Fowler et al., 1995). Virgin female NDL transgenic mice either homozygous for the Waved-2 mutation or nonwaved (either heterozygous for the waved-2 mutation or wild type genotype) were monitored by physical palpation for the appearance of mammary tumors. In contrast to the rapid tumor progression observed in the parental NDL strains, mammary tumors arose in both sets of Waved-2 genotypes with much longer onset and lower penetrance (Figure 11, Appendix 1). However despite the delayed tumor onset, no difference in the kinetics of tumor formation was observed in the either Waved-2 homozygotes or those possessing at least one functional copy of the EGFR (Figure 11, Appendix 1). To confirm that the *neu* transgene was expressed in these tumors, RNase protection analyses was conducted on RNA derived from tumors from a Waved-2 homozygote or nonwaved animal with transgene-specific riboprobe. The results of these analyses revealed that both tumors expressed equivalent levels of NDL transcript (Figure 12, Appendix 1).

To confirm whether the *neu* transcript encoded functional Neu, tissue extracts derived from tumors from Waved-2 homozygous and control *neu* transgenic mice were subjected to immunoprecipitation/immunoblot analyses with Neu and phosphotyrosine-specific antisera. As shown in Figure 13A, Appendix 1, tyrosine phosphorylated Neu could be detected in mammary tumors derived from either Waved-2 homozygotes or nonwaved *neu* transgenic mice (lanes 1-2, lanes 4-5, Figure 13A, Appendix 1) which correlated with the levels of Neu protein in these tissue (Figure 13B, Appendix 1). In contrast the normal adjacent mammary tissue expressed little Neu proteins (Figure 13A, lanes 3, 6, Appendix 1). This latter observation likely reflects the stochastic nature of transgene expression. Because Neu can transphosphorylate the endogenous EGFR, we also assessed the state of tyrosine phosphorylation of the EGFR in both normal and tumor tissues. As reported previously (Muller et al., 1996), variable levels of tyrosine phosphorylated EGFR were observed in the tumor and normal tissues from both Waved-2 homozygotes and nonwaved genotypes (Figure 13C, lanes 1-3, 4-6, Appendix 1) which again correlated with the levels of EGFR (Figure 13D, Appendix 1). However, activation of the EGFR was not closely correlated with expression of

activated Neu (compare Figure 13A with 13C, Appendix 1). Taken together these observations suggest that catalytic activation of the EGFR is dispensable for Neu-induced mammary tumorigenesis.

Expression of activated forms of the Neu/erbB-2 oncogene in transgenic mice results in the coordinate upregulation and activation of erbB-3 during mammary tumorigenesis.

One possible explanation for these observations is the EGFR/Neu heterodimer is not the critical heterodimer in Neu-induced mammary tumorigenesis. To explore this possibility further we examined whether other members of the EGFR family are co-expressed with Neu during mammary tumorigenesis. To accomplish this tumor extracts from several independent activated *neu* strains were subjected to immunoblot analyses with antisera for EGFR, erbB-3 and erbB-4 (Figure 14, Appendix 1). The results of these analyses revealed that amongst the EGFR family members, erbB-3 was expressed at elevated levels compared to either EGFR or erbB-4 in the Neu-induced mammary tumors (compare Figure 14B to 14A and 14C, Appendix 1). The coexpression of activated Neu and erbB-3 in these mammary tumors suggested that Neu could transactivate erbB-3. Given the fact that erbB-3 is catalytically inactive (Guy et. al., 1996), one indication of a functional erbB-3/Neu heterodimer is the presence of tyrosine phosphorylated erbB-3. To measure the state of erbB-3 phosphorylation protein lysates derived from the Neu-induced tumors were immunoprecipitated with erbB-3 specific antisera and immunoblotted with antiphosphotyrosine specific antisera. The results of these experiments showed that coexpression of activated Neu and erbB-3 resulted in the tyrosine phosphorylation of erbB-3 (Figure 15B, lanes 2-9, Appendix 1). These observations argue that Neu and erbB-3 are cooperating during mammary tumorigenesis through mechanism of transactivation.

To further explore the mechanism of upregulation of erbB-3 in these Neu-induced mammary tumors, we measured both the levels of erbB-3 transcript and protein in Neu -induced mammary tumors and adjacent normal mammary tissue. To evaluate the levels of erbB-3 transcript in the mammary tissues of these *neu* transgenic mice, RNA from both normal and tumor tissues were subjected to RNase protection analyses with riboprobes directed against erbB-3 cDNA. Quantitative measurement of the levels of erbB-3 transcript revealed that the levels of erbB-3 transcript did not vary between normal and tumor tissues (Figure 16, Appendix 1). The relative levels of expression of erbB-3 between these tissues could not be attributed to differences in epithelial content since the levels of epithelial keratin transcript were comparable. In contrast to these RNase protection analyses, quantitative immunoblot analyses with erbB-3 specific antibodies revealed a 10-20 fold increase in the steady state levels of erbB-3 protein (Figure 17, Appendix 1). Again these differences could not be attributed to differences in epithelial content since immunoblot analyses with keratin-8 antibodies indicated equivalent epithelial content (Figure 17, Appendix 1). Taken together, these findings suggest that the erbB-3 protein is dramatically upregulated during mammary tumor progression. Moreover, the mechanism that appears to be employed involves either upregulation of translation of erbB-3 transcripts or increased stability of of erbB-3 protein .

The studies outlined above suggest that erbB-3 is the principle heterodimeric partner for Neu in mammary tumorigenesis. To directly test the importance of erbB-3 and erbB-4 in mammary tumorigenesis, we have recently created transgenic mice that express erbB-3 and erbB-4 under the transcriptional control of the MMTV promoter/enhancer. To assess the pattern of transgene expression in these strains RNA derived from several tissues was subjected to RNase protection analyses with a transgene specific probe. Analyses of the tissue specificity of expression of several

independent MMTV/erbB-4 transgenic founder strains for each of these constructs revealed that low levels of erbB-4 transcript could be detected in the mammary epithelium (Figure 18, Appendix 1). Although these MMTV/erbB-4 mice have been maintained for over a year, none of these animals have yet developed mammary tumors. More recently we have derived comparable strains of transgenic mice expressing a MMTV/erbB-3 fusion gene. Preliminary expression analyses of tissue specificity of expression of the MMTV/erbB-3 strains has revealed expression of erbB-3 in virgin mammary epithelium and salivary glands (Figure 19, Appendix 1). Future crosses between these erbB-3 transgenic strains and MMTV/neu mice should provide important insight into the role of erbB-3 in tumor progression.

Conclusions

The results of our DOD sponsored research program have provided important insight into the role of the various EGFR family members in mammary tumorigenesis. Our first set of studies have focused on the role of the TGFα /EGFR axis in Neu-mediated tumorigenesis. We have demonstrated that coexpression of TGFα and Neu in mammary epithelium can result in a dramatic acceleration in the induction of mammary tumors. The rapid induction of mammary tumors in these strains was further correlated with elevated levels of tyrosine phosphorylated Neu (Figure 7D, Muller et al., 1996). However, inspection of the state of tyrosine phosphorylation of the EGFR revealed variable levels of phosphorylated EGFR (Figure 7B, Muller et al. 1996). One possible explanation for the variable levels of EGFR may reflect different ratios of the endogenous EGFR and the *neu* transgene. In this regard it is interesting to note that the mammary tumors arising in these bigenic strains either have a nodular or tubular phenotype (Figure 6, Muller et al., 1996). Whether these different tumor phenotypes reflect differential ratios of Neu and EGFR will require further studies. Nonetheless the results of these studies provide compelling evidence that Neu and TGFα can cooperate during mammary tumor progression.

Another interesting facet of these studies is the observation that in contrast to the parental *neu* transgenic strains, tumors derived from mice coexpressing Neu and TGFα failed to exhibit evidence of somatic mutations in the *neu* transgene (Figure 1A, Muller et al., 1996). Because these tumors exhibited elevated levels of tyrosine phosphorylated Neu, activation of Neu in these tumors is likely occurring through a mechanism involving transactivation of Neu by EGFR. More recently, we and our collaborators have demonstrated that a similar phenomenon occurs in transgenic mice coexpressing a mutant p53 allele and Neu in the mammary epithelium of transgenic mice. As with the bigenic TGFα /*neu* animals tumors arising in these strains failed to exhibit evidence of activating somatic mutations in the *neu* transgenes (Li et al., 1997). Again this is likely occurring through upregulation of TGFα in these tumors leading to transactivation of Neu by the activated EGFR (Li et al., 1997). While transactivation of the EGFR is a likely mechanism by which Neu is activated by EGFR, we have failed to detect stable complexes of EGFR and Neu by coimmunoprecipitation analyses (Figure 8, Muller et al., 1996). These observations argue that transactivation of Neu by EGFR is likely a transient process.

The results of these findings suggest that coexpression TGFα and Neu can cooperate to transform the mammary epithelium via transactivation of Neu by EGFR. One potential explanation for the accelerated tumor phenotype observed in the Neu/TGFα bigenic animals, is that both Neu and EGFR recruit distinct but complimentary signaling pathways that then cooperate to transform the mammary epithelium. Indeed, we have previously demonstrated that the c-Src family of tyrosine

kinases is preferentially recruited to Neu by in cells stimulated by specific EGFR family ligands (Muthuswamy and Muller, 1995). Consistent with this expectation, we have demonstrated that Neu is complexed with the Src family of tyrosine kinases in the TGFα /Neu bigenic tumors (Figure 9, Muller et al., 1996).

To further elucidate the importance of recruitment of c-Src by Neu upon stimulation by EGFR ligands, we have attempted to map the precise binding site for c-Src on Neu. To accomplish this, we have taken advantage of the inability of the EGFR to directly bind the Src family kinases. Using chimeric receptors between the EGFR and Neu, we have demonstrated that carboxyl segment of the catalytic domain of Neu (TK2 domain; Figure 3: Appendix 1) is both sufficient and necessary for mediating association of c-Src with Neu. One interesting aspect of these studies is that restoration of c-Src binding in these chimeric receptors as closely associated elevated basal levels of tyrosine kinase activity. It is conceivable that the associated tyrosine kinase activity in the absence of ligand stimulation is the result of the ability of c-Src to transactivate the chimeric receptor. In this regard it is noteworthy that previous studies have shown that Neu possesses a much higher basal level of tyrosine kinase activity (DiFiore et al., 1990). Given the preferential binding of c-Src to Neu, it is possible that elevated basal levels of Neu associated tyrosine kinase activity is due to its ability to selectively recruit the c-Src signaling pathway.

While the Src binding region on Neu has been localized to the carboxyl terminus of the Neu catalytic domain, the precise tyrosine residue responsible for binding of c-Src to Neu remains to identified. To map the c-Src binding site, we have created a number of point mutations in the four tyrosine residues in the TK2 domain (Figure 6, Appendix 1). While two of these mutations had little effect on Neu mediated transformation or c-Src binding (Figures 6 and 7, Appendix 1), two of these point mutations rendered Neu transformation defective and kinase inactive (Figure 7, Appendix 1). In fact, similar mutations in the Src binding sites of the platelet derived growth factor receptor (PDGFR) or colony stimulating factor-1 receptor (CSF-1R) resulted in severe impairment of kinase activity of these receptors (Courtneidge et al., 1993; Mori et al., 1993). Because both these mutations also dramatically effected Neu associated tyrosine kinase activity, the precise Src binding sites remains to be identified. To identify which of these two tyrosine residues is the c-Src binding site, we plan to synthesize phosphorylated peptides corresponding to each of these two remaining phosphorylation sites and determine whether they are capable of effectively compete with Neu for c-Src binding. Based on comparison of EGFR with Neu of the two candidate sites, only one is specific to Neu (Figure 6, Appendix 1 YHAD). Thus another means to demonstrate the importance of this site in mediating c-Src binding is to convert this site in the EGFR to the YHAD consensus observed in Neu. These future analyses should provide important insight into the role of c-Src in EGFR family signaling.

Whereas our observations suggest that under certain circumstances, activation of the EGFR can cooperate with Neu in transformation, whether EGFR function is essential for Neu mediated transformation is unclear. To investigate this possibility, we have interbred transgenic mice expressing an activated *neu* oncogene in the mammary epithelium (Figure 9, Appendix 1) with a naturally occurring mouse mutant that carries a kinase dead EGFR (Waved-2) (Luetteke et al., 1994; Fowler et al., 1995). One surprising outcome of these studies was the dramatic delay in tumor onset observed in the activated *neu* strains either in the presence or absence of the Waved-2 mutation. Although only 20% of the animals developed mammary tumors by a year of age in this mixed genetic background, no significant differences in the onset of mammary tumors was observed either Waved-2 homozygotes or their wild type counterparts (Figure 11 , Appendix 1). The delay in tumor

onset observed in these crosses could not be attributed to expression of the transgene since RNase protection analyses revealed the presence of the activated *neu* transcript (Figure 13, Appendix 1). One potential explanation for the observed delay in tumor formation is that introduction of the activated neu transgene into a different genetic background of the Waved-2 mice affected the onset of tumorigenesis. In fact, recent studies have demonstrated that genetic background can profoundly effect tumor onset in transgenic mice carrying a MMTV/neu transgene (Rowse et al., 1998). Whatever the explanation our observations strongly suggest that a functional EGFR is dispensable for Neu-mediated transformation of the mammary epithelium.

Although EGFR may be dispensable for Neu-induced mammary tumorigenesis, examination of these tumors for expression of other EGFR family members revealed that erbB-3 protein levels are upregulated during tumorigenesis (Figure 14, Appendix 1). Moreover we have demonstrated that erbB-3 is tyrosine phosphorylated (Figure 15, Appendix 1). Given the fact that erbB-3 is catalytically inactive (Guy et al., 1996), these observations suggest that transactivation of erbB-3 by activated Neu may be an important event in mammary tumorigenesis. One potential role erbB-3 may play in the induction of mammary tumors by Neu is to couple erbB-3 to the PI-3' kinase signaling pathway. Indeed, previous studies have demonstrated that erbB-3 is involved in recruiting the PI-3' kinase signaling molecule to Neu. Indeed, erbB-3 possesses 6 consensus binding sites for the PI-3' kinase (Sotloff et al., 1994, Pringent and Gullick, 1994). More recently, we have demonstrated that erbB-3 protein levels are dramatically upregulated in tumors arising in transgenic mice expressing a mutant Polyomavirus middle T (PyV mT) incapable of associating the PI-3' kinase (Webster et al., 1998). Based on these observations, we hypothesize that the upregulation of erbB-3 in this tumor model can indirectly compensate for the inability of the mutant PyV mT to associate with the PI-3' kinase. Taken together, these observations suggest that upregulation of erbB-3 protein may be frequent event during mammary tumorigenesis.

Another interesting aspect of these studies is the observation that the upregulation of erbB-3 is occurring at either translational or postranslational level since the levels of erbB-3 transcript levels are equivalent between normal and tumor tissues (Figure 16, Appendix 1). In this regard it interesting to note that activation of the PI-3' /Akt kinase has been implicated in upregulation of translation by interfering with inhibitory translational subunit (Gingras et al., 1998). Thus the upregulation of erbB-3 levels may occur through stimulation of the PI-3' kinase signaling pathway. In addition to affecting translation, activation of the PI-3' kinase signaling pathway also plays an important role in preventing apoptosis (Webster et al., 1998). Indeed we have demonstrated that expression of mutant PyV mT oncogene incapable of associating with the PI-3' kinase leads to widespread hyperplasias that are highly apoptotic (Webster et al., 1998). It is conceivable that upregulation of erbB-3 during Neu mediated tumorigenesis plays an important role in recruiting the PI-3' kinase pathway which in turn activates an antiapoptotic program.

To directly assess the importance of erbB-3 in mammary tumorigenesis, we have recently generated trangenic mice that express erbB-3 in the mammary epithelium (Figure 19, Appendix 1). Preliminary analyses of the tissue specificity of expression of seven independent founder animals with a transgene specific probe revealed that one of these expressed elevated erbB-3 transcripts in the mammary epithelium (Figure 19, Appendix 1). To further assess the importance of erbB-3 in Neu induced mammary tumors, we plan to interbreed these transgenic mice with the activated neu transgenic strains. The results of these studies should provide important insight into the role of erbB-3 in mammary tumorigenesis.

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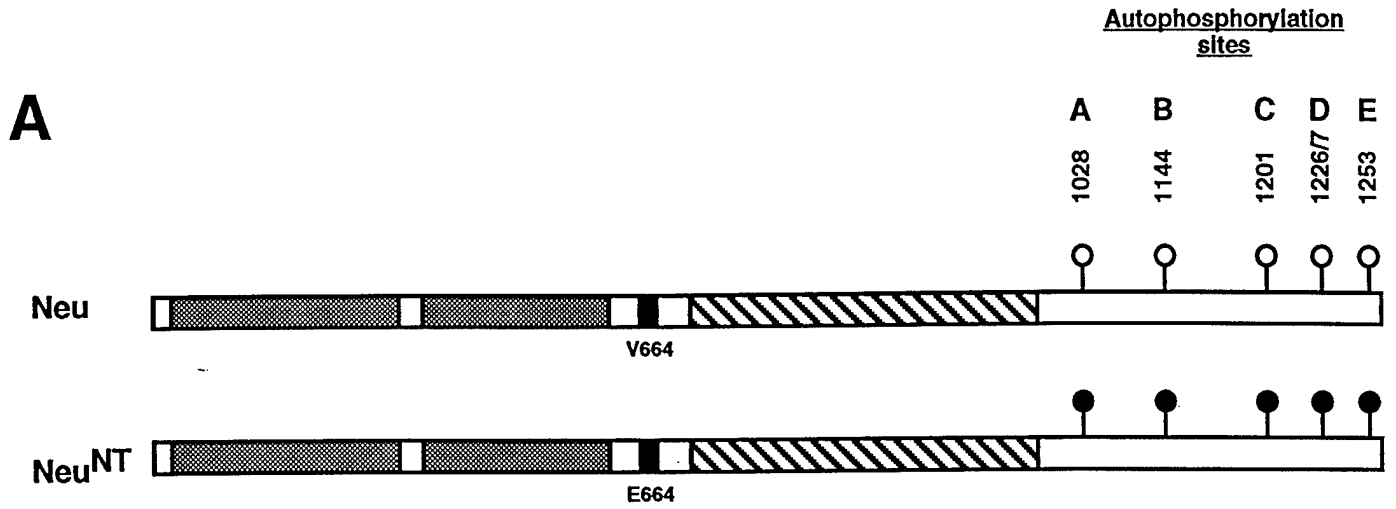
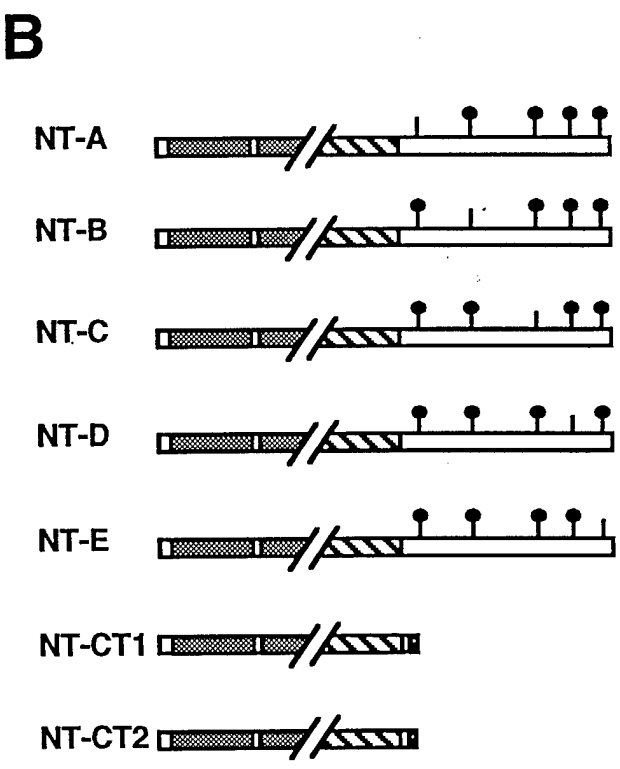
Figure. 1 Schematic representation of Neu receptor kinase mutants. (**A**) The structure of Neu and activated Neu (Neu ^{NT}), containing the transmembrane mutation V664E, are depicted. Indicated are two cysteine rich domains (shaded), a single transmembrane domain (black) and the tyrosine kinase domain (striped). Autophosphorylation sites at tyrosine residues 1028 (site A), 1144 (site B), 1201 (site C), 1226/7(site D) and 1253 (site E) are indicated (Y). (**B**) Indicated tyrosine residues were converted to phenylalanine residues (F). NT-CT1 carries a stop codon immediately following the methionine codon at 1005 and NT-CT2 is a frame shift deletion mutant terminating with the sequences ^{1005}MHGQYLLPFTAGR, where the underlined amino acids differ from that of Neu. (**C**) NT-NYPD contains mutations at each of the indicated autophosphorylation sites. Add-back mutants derived from NT-NYPD contain single autophosphorylation sites and phenylalanine residues at the four remaining sites. All mutants were derived from Neu^{NT}.


A
Autophosphorylation sites
A
B
C
D
E
1028
1144
1201
1226/7
1253
Neu
V664
NeuNT
E664


B
NT-A
NT-B
NT-C
NT-D
NT-E
NT-CT1
NT-CT2

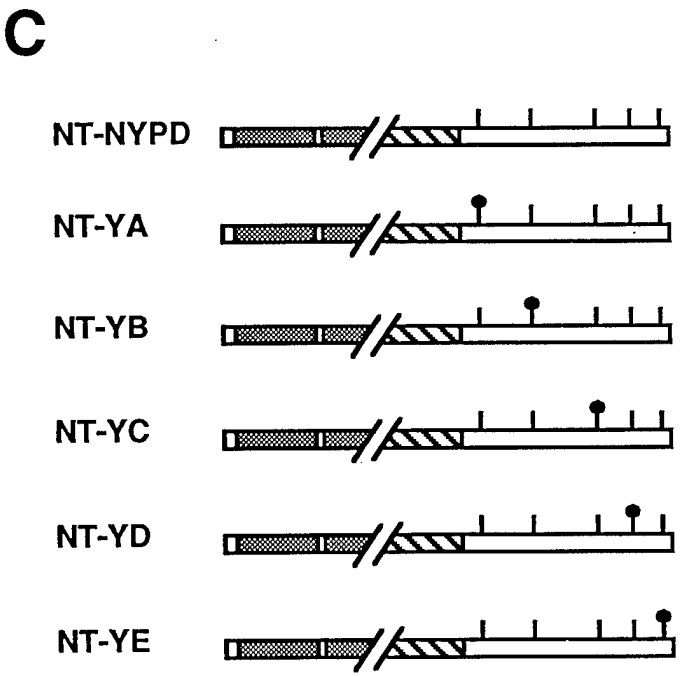

C
NT-NYPD
NT-YA
NT-YB
NT-YC
NT-YD
NT-YE

Figure 2 Tyrosine-to-phenylalanine point mutations of the five autophosphorylation sites on Neu do not mediate c-Src association to the receptor. **a)** *In vitro* analysis of single point mutants or add-back mutants of the five autophosphorylation sites on Neu were analyzed to address whether the SrcSH2 fusion protein can associate with these sites. **b)** The association of the SrcSH2 fusion protein was not dependent on variations in the levels of tyrosine phosphorylated Neu. **c)** *In vivo* analysis via coimmunoprecipitation with c-Src reveals that the five autophosphorylation sites do not mediate c-Src binding to Neu. **d)** The association between NYPD and c-Src correlates with a high level of tyrosine phosphorylated receptor.

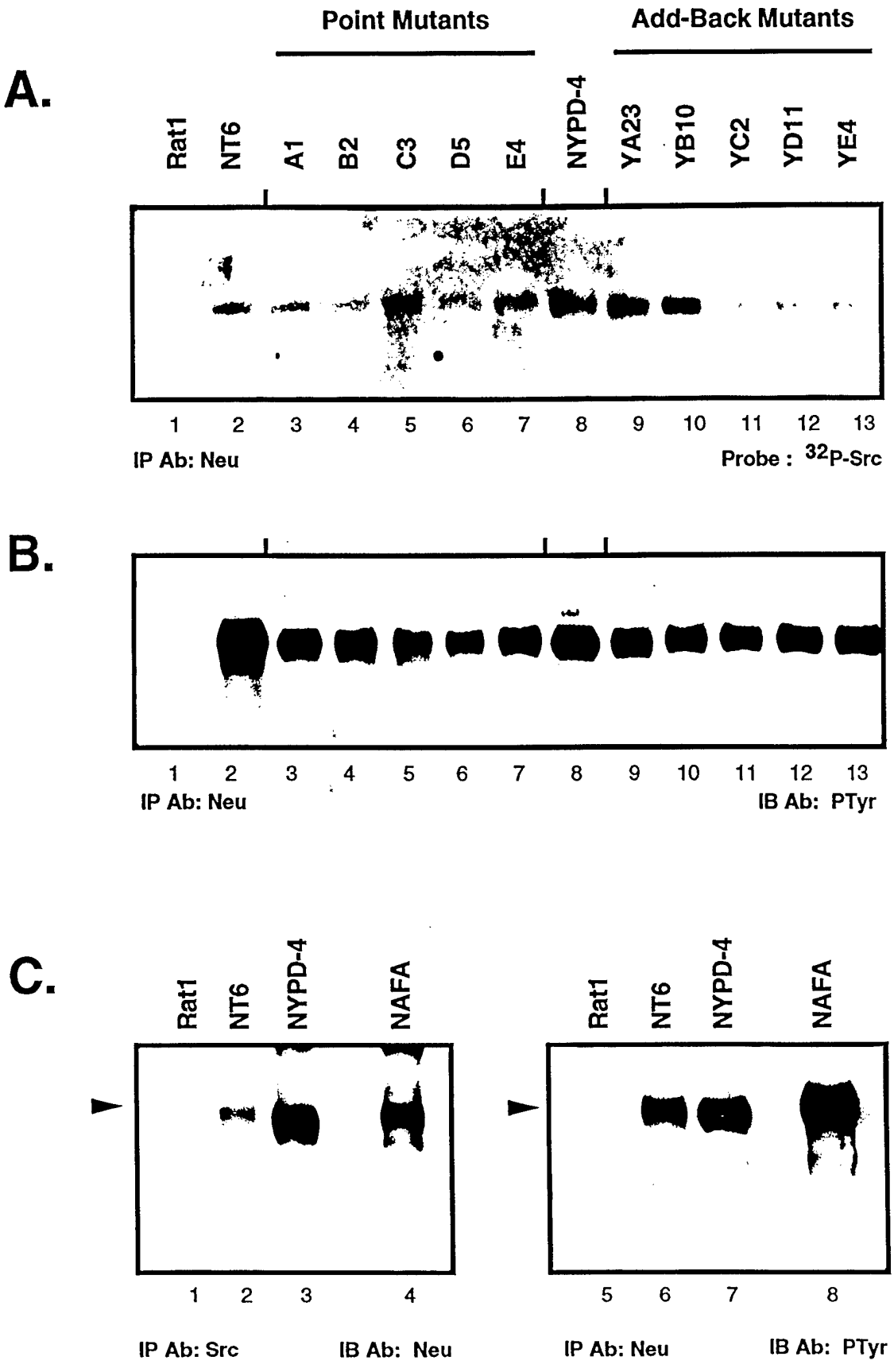

A.
Point Mutants
Add-Back Mutants
Rat1
NT6
A1
B2
C3
D5
E4
NYPD-4
YA23
YB10
YC2
YD11
YE4
1 2 3 4 5 6 7 8 9 10 11 12 13
IP Ab: Neu
Probe : 32P-Src
B.
1 2 3 4 5 6 7 8 9 10 11 12 13
IP Ab: Neu
IB Ab: PTyr
C.
Rat1
NT6
NYPD-4
NAFA
Rat1
NT6
NYPD-4
NAFA
1 2 3 4
5 6 7 8
IP Ab: Src
IB Ab: Neu
IP Ab: Neu
IB Ab: PTyr

Figure 3. Diagrammatic representation of EGFR/c-ErbB2 chimeric constructs. Expression of wild type EGFR and the chimeric receptors are driven by the molony murine leukemia virus LTR. Stable cell lines in Rat 1 fibroblasts were established by calcium phoshpate co-transfection.

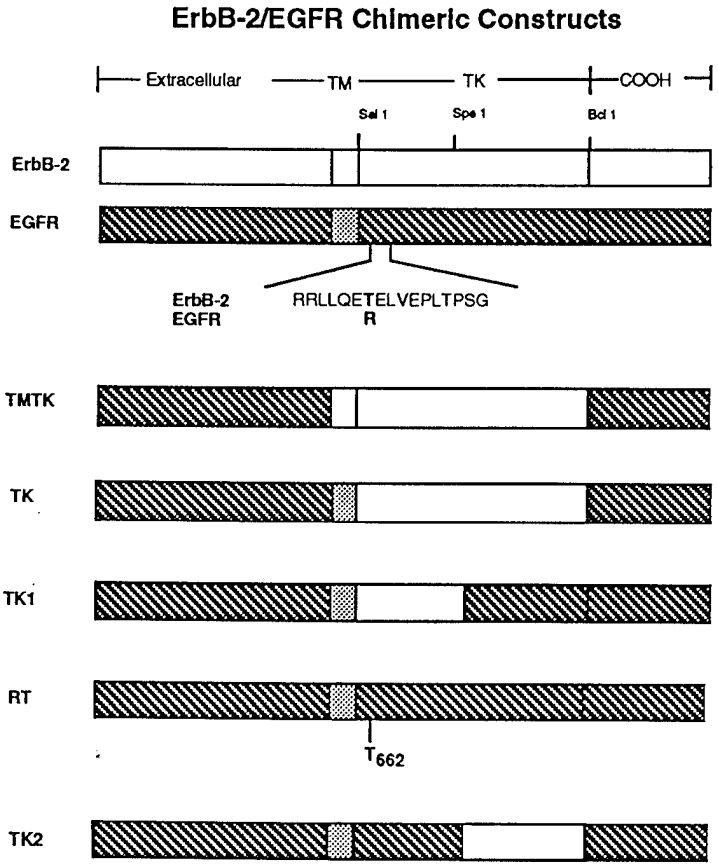
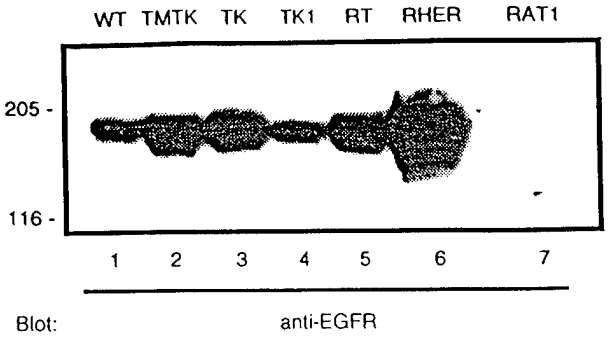
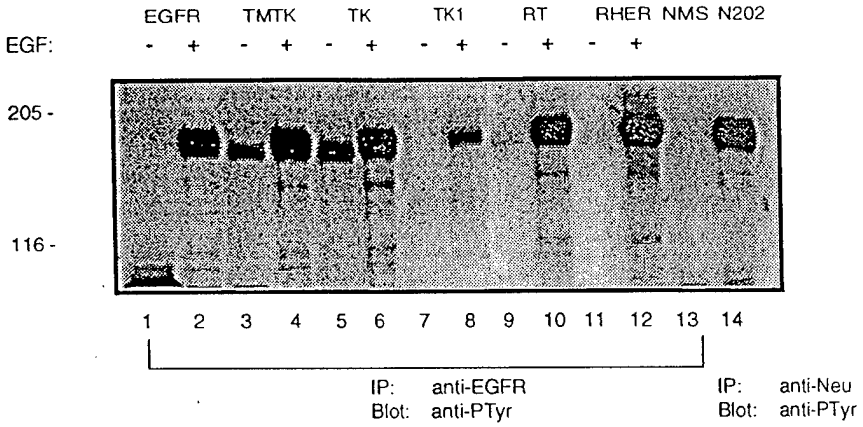
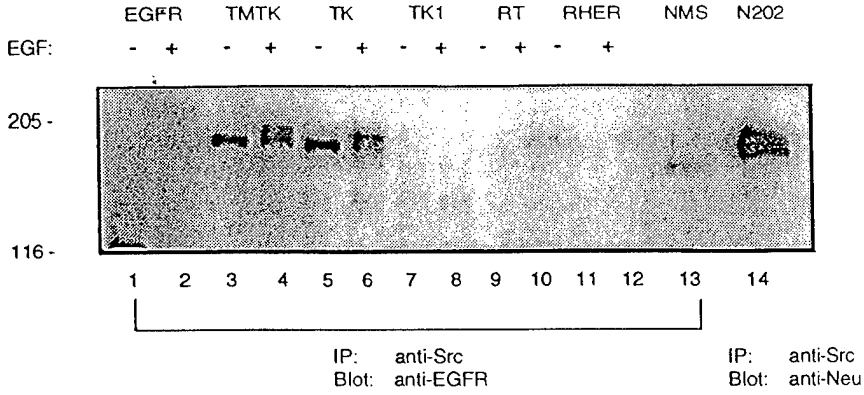

ErbB-2/EGFR Chimeric Constructs
Extracellular
TM
TK
COOH
Sal 1
Spe 1
Bcl 1
ErbB-2
EGFR
ErbB-2
EGFR
RRLLQETELVEPLTPSG
R
TMTK
TK
TK1
RT
T662
TK2

Figure 4. *In vivo* association of c-Src with the receptor chimerics. **a)** Stable cell lines expressing the various chimerics were induced with EGF and **b)** activation was assessed by measuring receptor tyrosine phosphorylation. **c)** c-Src co-immunoprecipitates specifically with TMTK and TK. **d)** The differential association of c-Src to the chimeras was not dependent on the levels of c-Src. Positive control was N202 mammary tumor tissue. Co-immunoprecipitation analysis of stable cell lines on confluent (150mm) plates were performed following EGF stimulation (100ng/ml) for 5 minutes. All plates were washed twice in ice cold PBS with 1mM sodium orthovanadate and lysed on ice with 0.7% 3-{(cholamidopropyl)-dimethyl-ammonio}-1-propaneosulphonate (CHAPS) lysis buffer (50 mM Tris HCl pH 8.0, 0.7% CHAPS, 50mM NaCl, 1 mM sodium orthovanadate, 10ug/ml leupeptin, 10ug/ml aprotinin). Immunoprecipitations were performed by incubating 1.5 mg of total cell lysate with 2-3 ug of anti-Src (7D10, Quality Biotech) or NMS and 40 ul of protein G sepharose for 3 hours rotating at 4^{o}C and subsequently washed 4 times with 0.7% CHAPS lysis buffer. Samples were boiled for 5 minutes in 40 ul of 1x SDS loading buffer and fractionated by SDS-PAGE. Immunoblotting of PVDF membranes with anti-EGFR (Transduction Laboratories), anti-Neu (7.16.4), anti-Phosphotyrosine (Transduction Laboratories) or anti-Src (Santa Cruz) was performed via standard techniques.


WT TMTK TK TK1 RT RHER RAT1
205 -
116 -
1 2 3 4 5 6 7
Blot: anti-EGFR


EGFR TMTK TK TK1 RT RHER NMS N202
EGF: - + - + - + - + - + - +
205 -
116 -
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IP: anti-EGFR
Blot: anti-PTyr
IP: anti-Neu
Blot: anti-PTyr


EGFR TMTK TK TK1 RT RHER NMS N202
EGF: - + - + - + - + - + - +
205 -
116 -
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IP: anti-Src
Blot: anti-EGFR
IP: anti-Src
Blot: anti-Neu

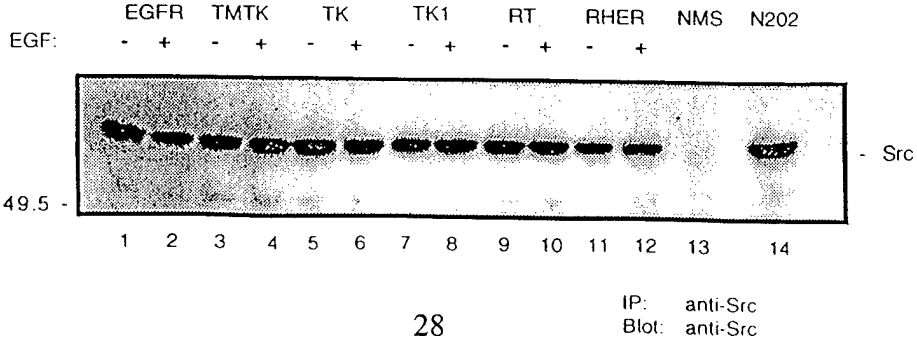
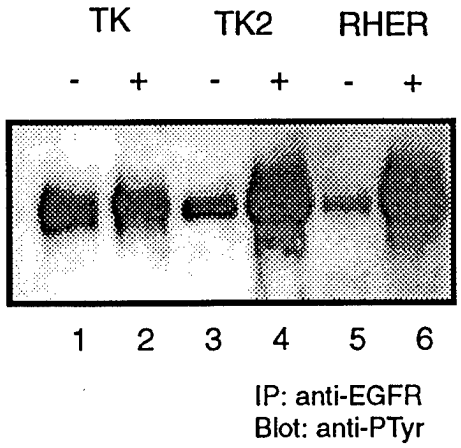
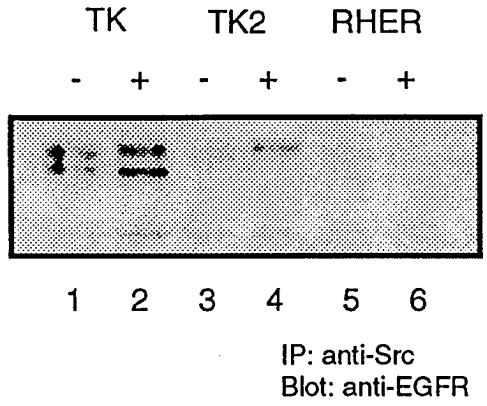

EGFR TMTK TK TK1 RT RHER NMS N202
EGF: - + - + - + - + - + - +
49.5 -
Src
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IP: anti-Src
Blot: anti-Src

Figure 5. *In vivo* association of c-Src with TK2. **a)** Chimeric receptors TK, TK2 and wild-type EGFR expressed in the cell line RHER were stimulated with EGF and receptor tyrosine phosphorylation was assessed. **b)** c-Src specifically co-immunoprecipitates with the chimeric that contains the TK2 region of the c-ErbB2 kinase domain. **c)** The difference in association was not due to differences in the levels of c-Src. Co-immunoprecipitation analysis of stable cell lines on confluent (150mm) plates were performed following EGF stimulation (100ng/ml) for 5 minutes. All plates were washed twice in ice cold PBS with 1mM sodium orthovanadate and lysed on ice with 0.7% 3-{(cholamidopropyl)-dimethyl-ammonio}-1-propaneosulphonate (CHAPS) lysis buffer (50 mM Tris HCl pH 8.0, 0.7% CHAPS, 50mM NaCl, 1 mM sodium orthovanadate, 10ug/ml leupeptin, 10ug/ml aprotinin). Immunoprecipitations were performed by incubating 1.5 mg of total cell lysate with 2-3 ug of anti-Src (7D10, Quality Biotech) and protein G sepharose for 3 hours rotating at 4^{o}C and subsequently washed 4 times with 0.7% CHAPS lysis buffer. Samples were boiled for 5 minutes in 1x SDS loading buffer and seperated by SDS-PAGE. Immunoblotting of PVDF membranes with anti-EGFR (Transduction Laboratories), anti-Phosphotyrosine (Transduction Laboratories) or anti-Src (Santa Cruz) was performed via standard techniques.

A.


TK
TK2
RHER
EGF: - + - + - +
1 2 3 4 5 6
IP: anti-EGFR
Blot: anti-PTyr

B.


TK
TK2
RHER
EGF: - + - + - +
1 2 3 4 5 6
IP: anti-Src
Blot: anti-EGFR

C.

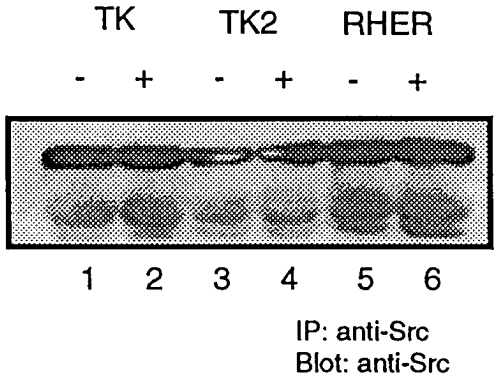
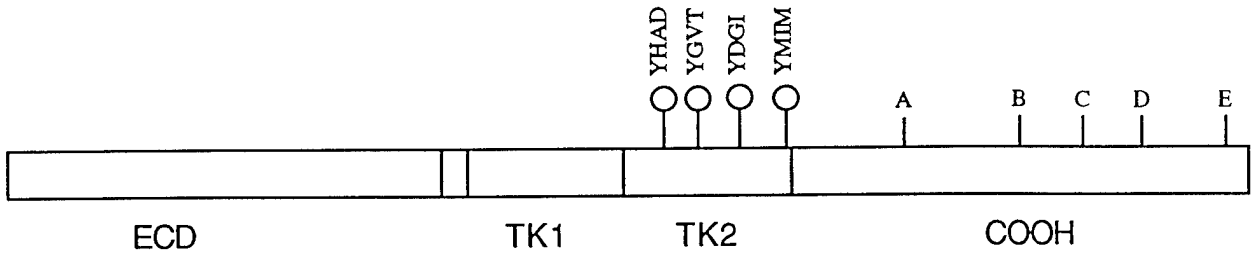

TK
TK2
RHER
EGF: - + - + - +
1 2 3 4 5 6
IP: anti-Src
Blot: anti-Src

Figure 6 Tyrosine-to phenylalanine mutations within the TK2 region confers differences in transformation. **a)** Tyrosines that are found in the TK2 region of the Neu receptor tyrosine kinase were mutated to phenylalanine. **b)** Foci were scored over 5 plates and normalized to puromycin resistant colonies. Comparisons were relative to activated Neu.

A


YHAD
YGVT
YDGI
YMIM
A
B
C
D
E
ECD
TK1
TK2
COOH

B

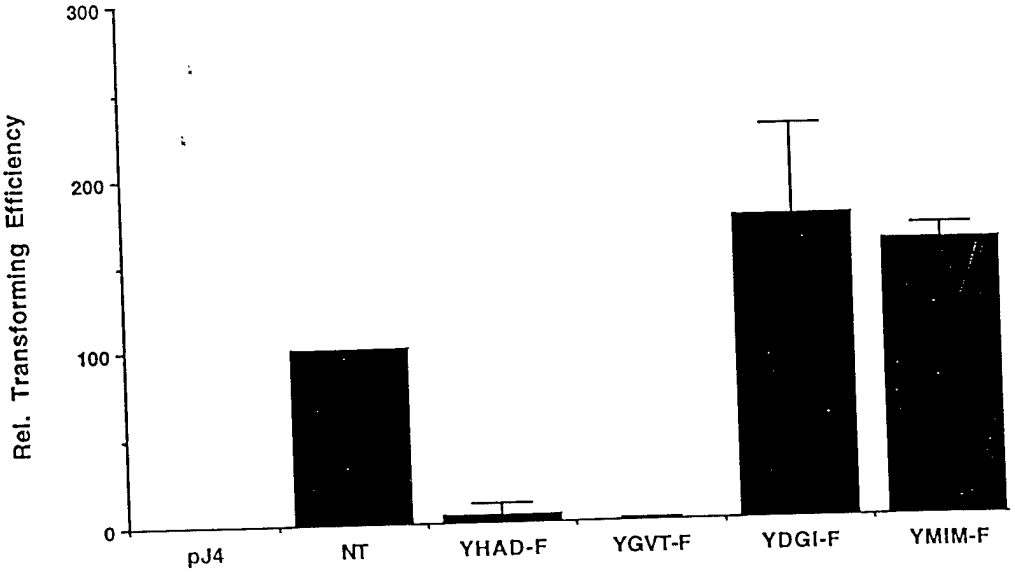
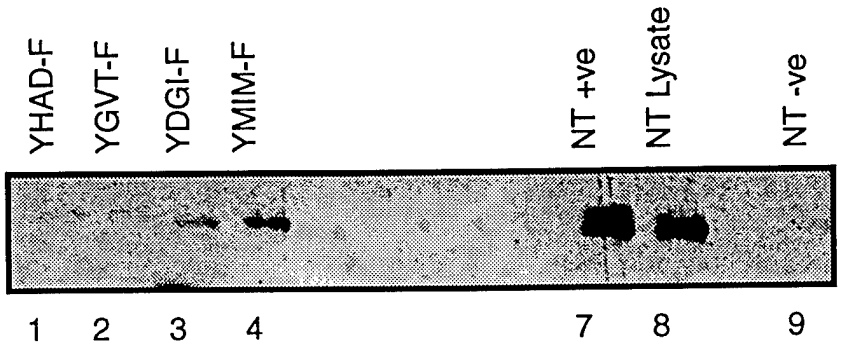
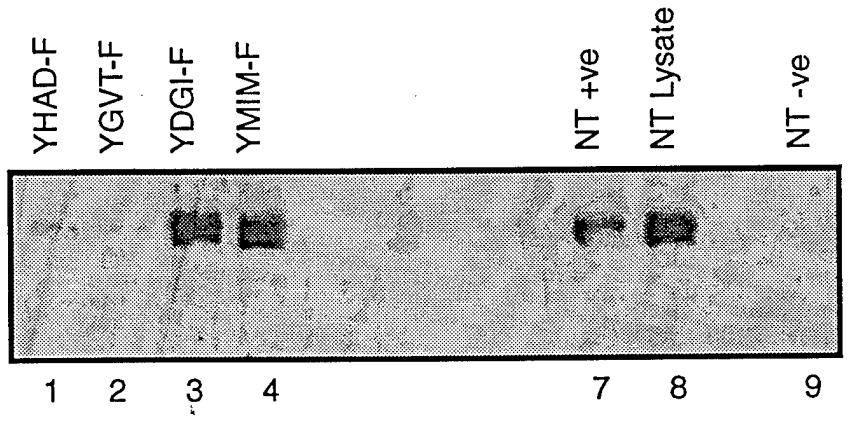

Rel. Transforming Efficiency
300
200
100
0
pJ4
NT
YHAD-F
YGVT-F
YDGI-F
YMIM-F

Figure 7. *In vivo* association of c-Src with tyrosine-to-phenylalnine point mutations within the TK2 region. **a)** Stable cell lines that express each point mutant was stimulated with EGF and receptor tyrosine phosphorylation was assessed. **b)** c-Src specifically co-immunoprecipitates with YDGI-F and YMIM-F but not with YHAD-F or YGVT-F. **c)** The difference in association was not due to differences in the levels of c-Src. Co-immunoprecipitation analysis of stable cell lines on confluent (150mm) plates were performed following two washes with ice cold PBS containing 1mM sodium orthovanadate, and lysed on ice with 0.7% 3-{(cholamidopropyl)-dimethyl-ammonio}-1-propaneosulphonate (CHAPS) lysis buffer (50 mM Tris HCl pH 8.0, 0.7% CHAPS, 50mM NaCl, 1 mM sodium orthovanadate, 10ug/ml leupeptin, 10ug/ml aprotinin). Immunoprecipitations were performed by incubating 1.5 mg of total cell lysate with 2-3 ug of anti-Src (7D10, Quality Biotech) and protein G sepharose for 3 hours rotating at $4^{o}C$ and subsequently washed 4 times with 0.7% CHAPS lysis buffer. Samples were boiled for 5 minutes in 1x SDS loading buffer and seperated by SDS-PAGE. Immunoblotting of PVDF membranes with anti-Neu (Oncogene Science), anti-Phosphotyrosine (Transduction Laboratories) or anti-Src (Santa Cruz) was performed via standard techniques.

A.


YHAD-F
YGVT-F
YDGI-F
YMIM-F
NT +ve
NT Lysate
NT -ve
1 2 3 4 7 8 9
IP: anti-Src
Blot: anti-Neu

B.


YHAD-F
YGVT-F
YDGI-F
YMIM-F
NT +ve
NT Lysate
NT -ve
1 2 3 4 7 8 9
IP: anti-Neu
Blot: anti-PTyr

C.

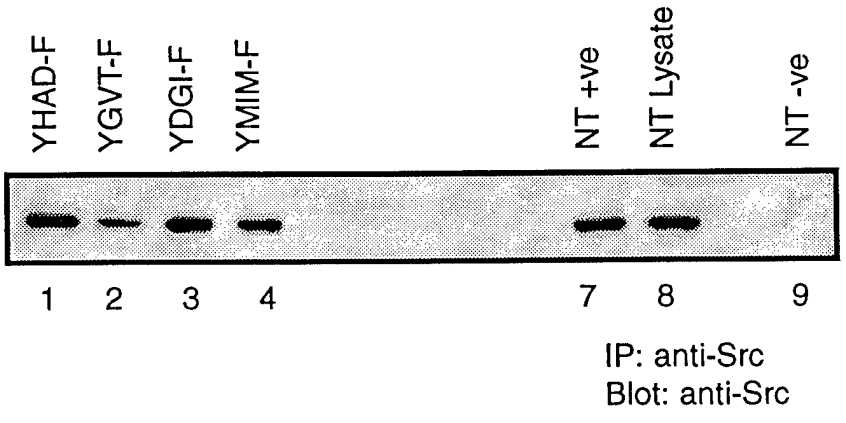

YHAD-F
YGVT-F
YDGI-F
YMIM-F
NT +ve
NT Lysate
NT -ve
1 2 3 4 7 8 9
IP: anti-Src
Blot: anti-Src

FIG. 8. Kinetics of tumor occurrence in NDL transgenic mouse strains. Comparison of the kinetics of tumor formation between virgin female carriers of the best characterized lines from both the NDL1 and NDL2 strains (NDL1-2 and NDL2-5) with virgin females from a wild-type *neu* expressing strain (N202). The number of mice examined (n) and the age at which 50% of the animals were found to have tumors (t50) in each line is indicated.

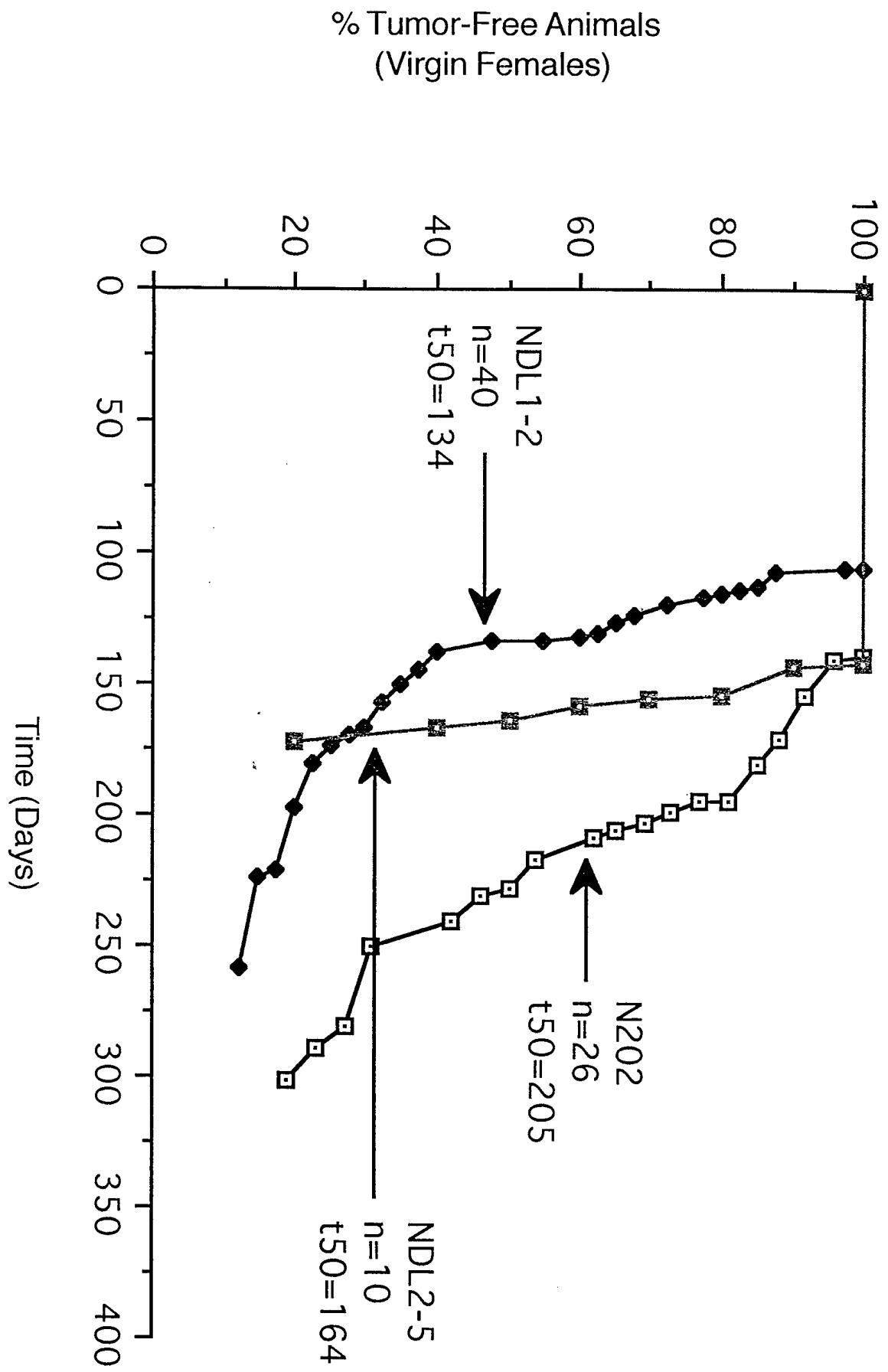
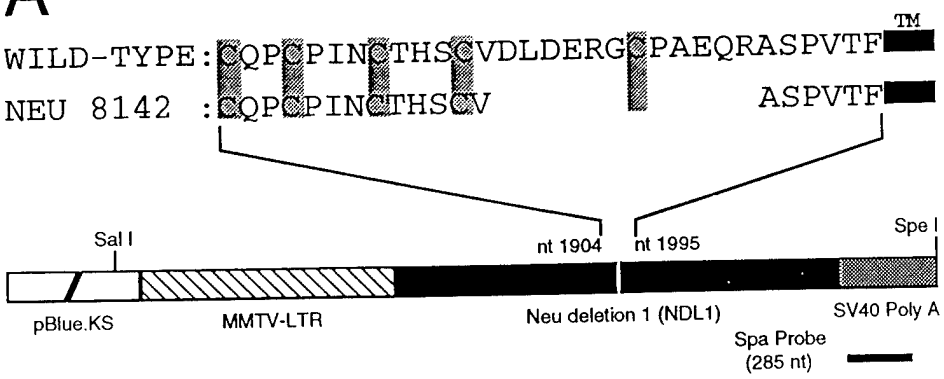
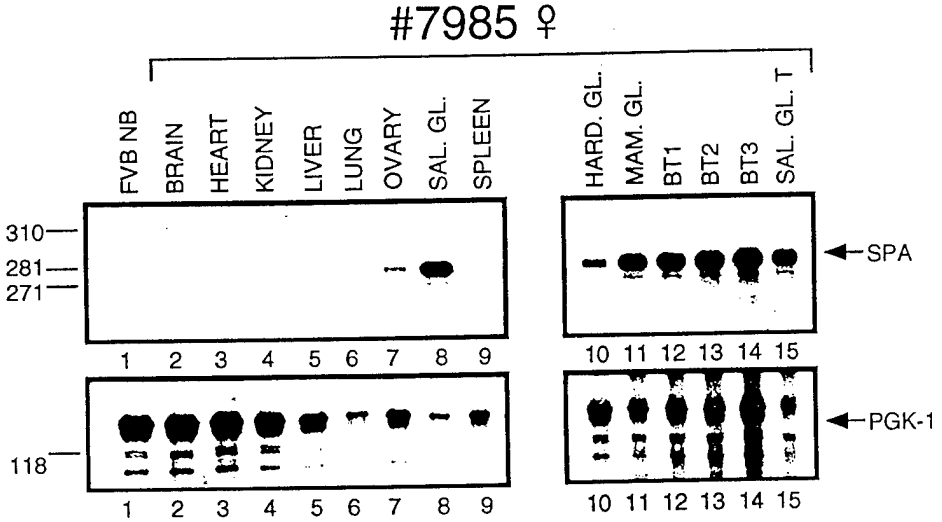

% Tumor-Free Animals
(Virgin Females)
0
20
40
60
80
100
0
50
100
150
200
250
300
350
400
Time (Days)
NDL1-2
n=40
t50=134
N202
n=26
t50=205
NDL2-5
n=10
t50=164

FIG. 9. Structure of *neu* deletion-1 (NDL1) transgene and tissue specificity of transgene expression. (A) Transgene structure. The unshaded region indicates vector sequences (pBluescript KS), the striped region represents the MMTV-LTR, filled regions correspond to the *neu* cDNA which harbors a deletion, and the grey portion indicates polyadenylation signals derived from the SV40 early transcription unit. The amino acid sequence of the deletion mutation encoded by this cDNA is compared to the wild-type sequence and is outlined in single letter code above the transgene schematic. The Spa (SV40 polyadenylation) riboprobe used for the RNase protection analyses presented in (B) and (C) is also indicated. (B) RNA transcripts corresponding to the MMTV/NDL1 transgene in various organs of a female carrier (#7985) from the NDL1-2 line. This female was multiparous (n=3) and 118 days of age at the time of autopsy. The fragment protected by the antisense riboprobe is marked by SPA and an arrow. A riboprobe directed against the mouse phosphoglycerate kinase gene was also included in the hybridizations to control for equal loading of RNA and is marked by PGK-1 and an arrow. (C) RNA transcripts corresponding to the MMTV/NDL1 transgene in various organs of a male carrier (#7991) from the NDL1-2 line. This male was 213 days of age at the time of autopsy. The riboprobes used to conduct this RNase protection are the same as those described in (B). Migration of DNA markers is indicated (nucleotides) on the left of each panel found in (B) and (C). NB, normal breast; Sal. Gl., salivary gland; Hard. Gl., harderian gland; Mam. Gl., mammary gland; BT, breast tumor; Sal. Gl. T., salivary gland tumor; Sem. Ves., seminal vesicles.

A


WILD-TYPE:CQPCPINCTHSCVDLDERGCPAEQRASPVTF
TM
NEU 8142 :CQPCPINCTHSCV
ASPVTF
Sal I
nt 1904
nt 1995
Spe I
pBlue.KS
MMTV-LTR
Neu deletion 1 (NDL1)
SV40 Poly A
Spa Probe
(285 nt)

B


#7985 ♀
FVB NB
BRAIN
HEART
KIDNEY
LIVER
LUNG
OVARY
SAL. GL.
SPLEEN
HARD. GL.
MAM. GL.
BT1
BT2
BT3
SAL. GL. T
310
281
271
118
SPA
PGK-1
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15

C

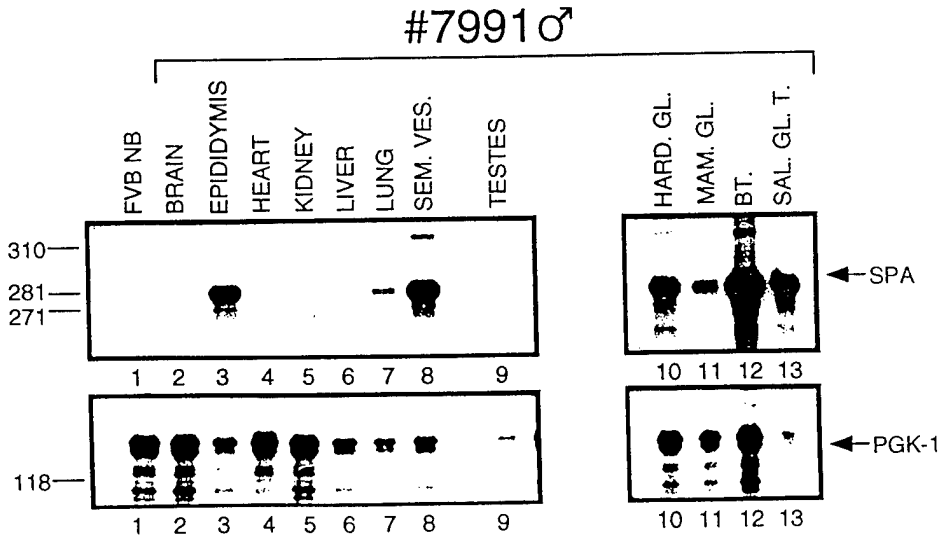
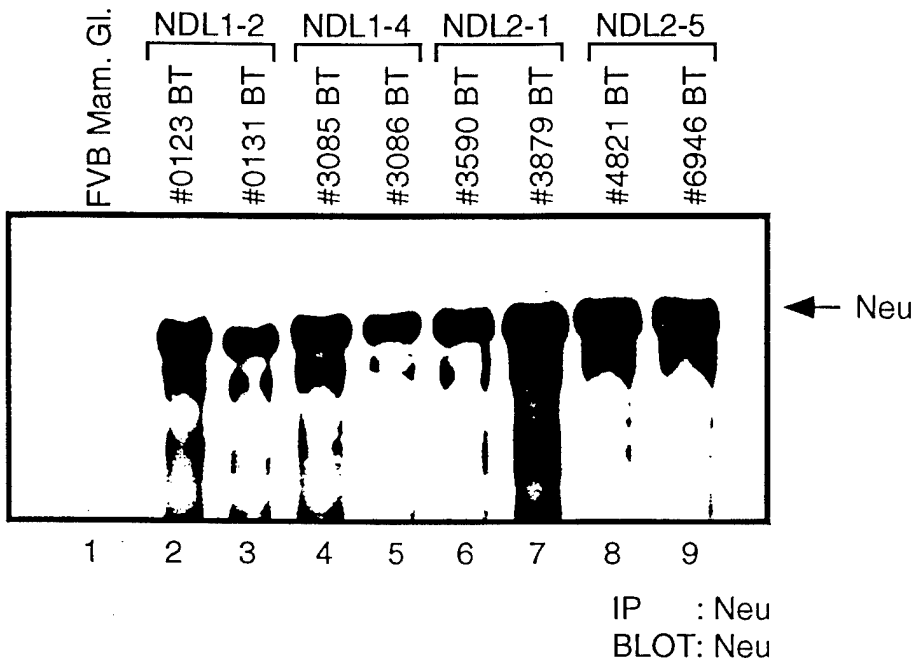

#7991 ♂
FVB NB
BRAIN
EPIDIDYMIS
HEART
KIDNEY
LIVER
LUNG
SEM. VES.
TESTES
HARD. GL.
MAM. GL.
BT.
SAL. GL. T.
310
281
271
118
SPA
PGK-1
1 2 3 4 5 6 7 8 9
10 11 12 13

FIG. 10. Altered Neu receptors encoded by both MMTV/*neu* deletion transgenes (NDL1 and NDL2) are constitutively tyrosine phosphorylated. (A) Neu was immunoprecipitated (IP) from mammary tumor lysates (BT) derived from each of four different transgenic lines expressing Neu deletion mutants (lanes 2 to 9). Tumor tissue was collected from two separate transgenic strains (NDL1 and NDL2) and two independent lines were examined from each strain (NDL1-2, NDL1-4 and NDL2-1, NDL2-5). One-third of the immunoprecipitate was electrophoresed on an SDS-9.0% polyacrylamide gel, transferred to a PVDF membrane, and subjected to immunoblot analysis (BLOT) with a Neu specific antibody. The position of Neu is indicated by the arrow. (B) The remaining two-thirds of the immunoprecipitate was subjected to immunoblot analysis for anti-phosphotyrosine (P-Tyr) as described in (A). The position of tyrosine phosphorylated Neu is indicated by the arrow. A lysate from non-transgenic mammary tissue (FVB Mam. Gl.) was included as a negative control (lane 1).

A


FVB Mam. Gl.
NDL1-2
NDL1-4
NDL2-1
NDL2-5
#0123 BT
#0131 BT
#3085 BT
#3086 BT
#3590 BT
#3879 BT
#4821 BT
#6946 BT
Neu
1 2 3 4 5 6 7 8 9
IP : Neu
BLOT: Neu

B

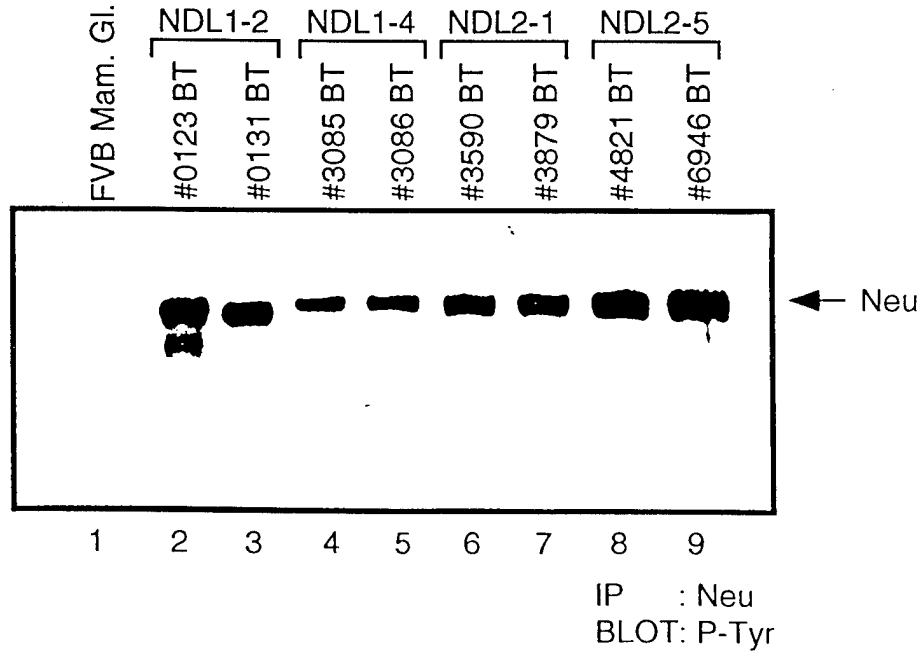

FVB Mam. Gl.
NDL1-2
NDL1-4
NDL2-1
NDL2-5
#0123 BT
#0131 BT
#3085 BT
#3086 BT
#3590 BT
#3879 BT
#4821 BT
#6946 BT
Neu
1 2 3 4 5 6 7 8 9
IP : Neu
BLOT: P-Tyr

FIG.11. Mammary tumor onset in wavy/NDL and not wavy/NDL mice

Comparison of mammary tumor incidence between wavy/NDL and not wavy/NDL mice. The number of mice examined in each group are indicated.

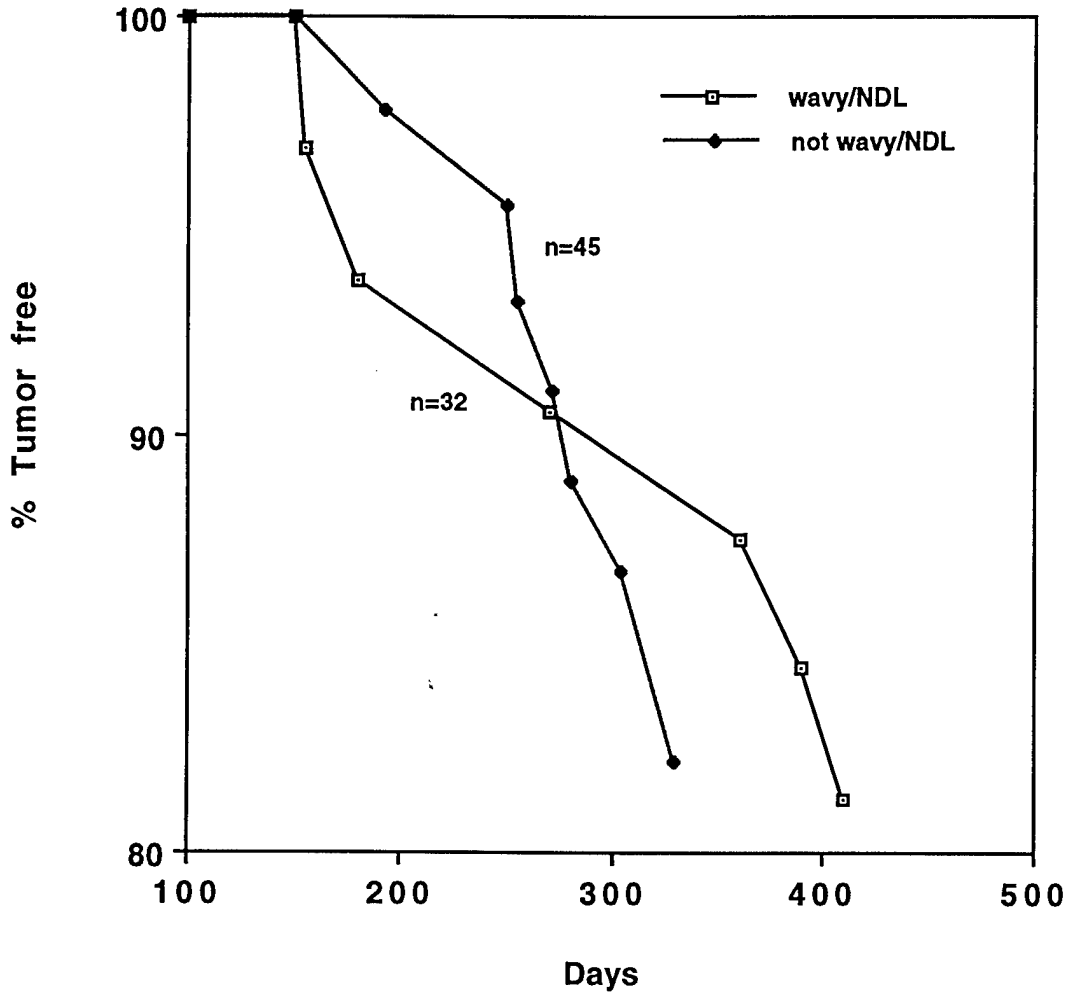

100
90
80
% Tumor free
100
200
300
400
500
Days
wavy/NDL
not wavy/NDL
n=45
n=32

FIG. 12. Phenotypic comparison and expression of NDL transcripts in wavy/NDL and not wavy/NDL mice

(A) Phenotypic comparison of NDL transgenic mice heterozygous to homozygous for the wa-2 mutation. Both mice are 231 days of age. (B) RNase protection analysis of expression of NDL transcripts in wa-2/+ and wa-2/wa-2 mammary epithelium. RNA was isolated from tissue derived from each of the above mice and hybridized against the antisense rat neu riboprobe. An antisense probe directed against the mouse phosphoglycerate kinase gene (PGK-1) was used as control for equal loading of RNA on the gel.

A

ndl-1/+; wa-2/+

ndl-1/+; wa-2/wa-2

B

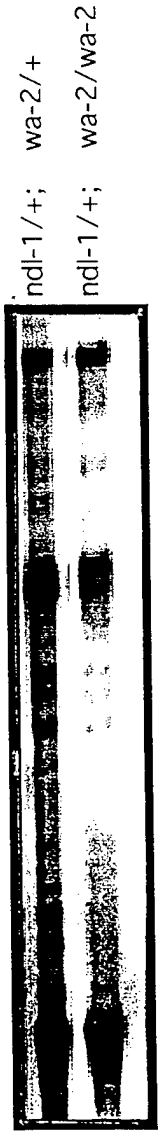
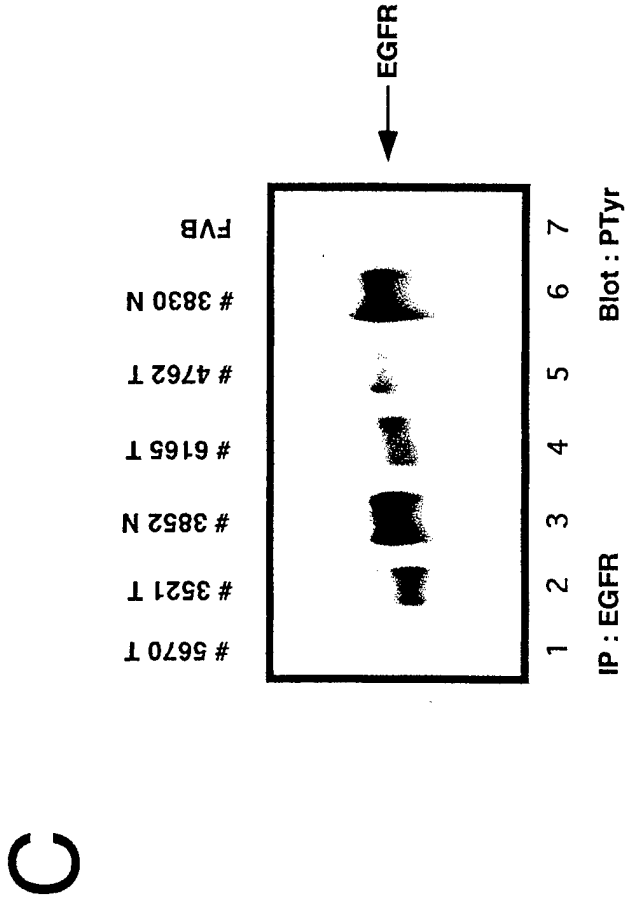
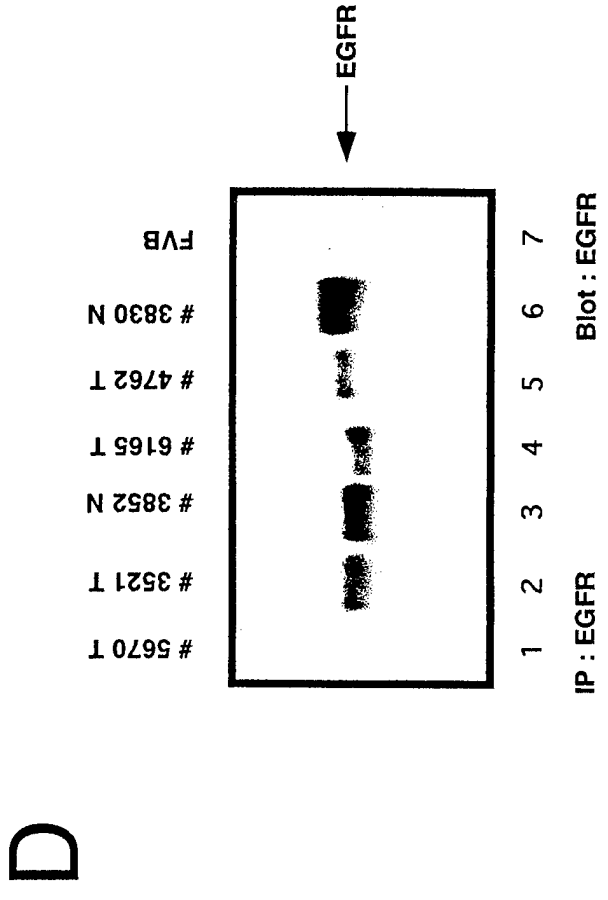
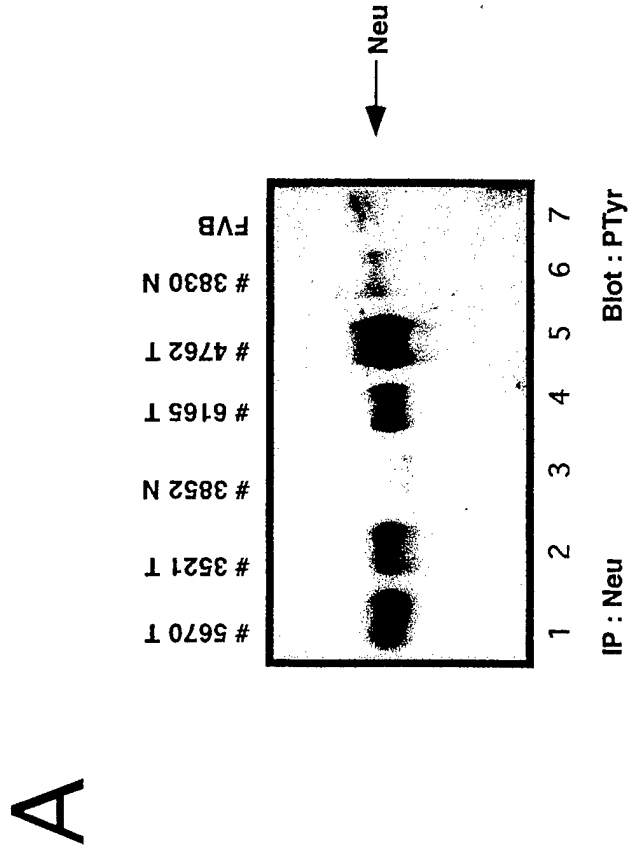


FIG. 13. Tyrosine phosphorylation of Neu and EGFR in tumors from not wavy NDL and wavy/NDL mice.

Protein lysates from tumor (T) or normal (N) tissue from not wavy/NDL, wavy/NDL or normal FVB female were immunoprecipitated (IP) for Neu (monoclonal antibody 7.16.4) and then immunoblotted with either antiphosphotyrosine antibody (PY20) (A) or a Neu specific antibody (Ab3, Oncogene Sciences) (B). In panels (C) and (D) protein lysates similar to those in panel (A) were immunoprecipitated with an anti-EGFR antibody and then subjected to immunoblot analysis with PY20 antibody or anti-EGFR antiserum respectively. The positions of Neu and EGFR are indicated by arrows.


C
EGFR
FVB
3830 N
4762 T
6165 T
3852 N
3521 T
5670 T
1 2 3 4 5 6 7
IP : EGFR
Blot : PTyr


D
EGFR
FVB
3830 N
4762 T
6165 T
3852 N
3521 T
5670 T
1 2 3 4 5 6 7
IP : EGFR
Blot : EGFR


A
Neu
FVB
3830 N
4762 T
6165 T
3852 N
3521 T
5670 T
1 2 3 4 5 6 7
IP : Neu
Blot : PTyr

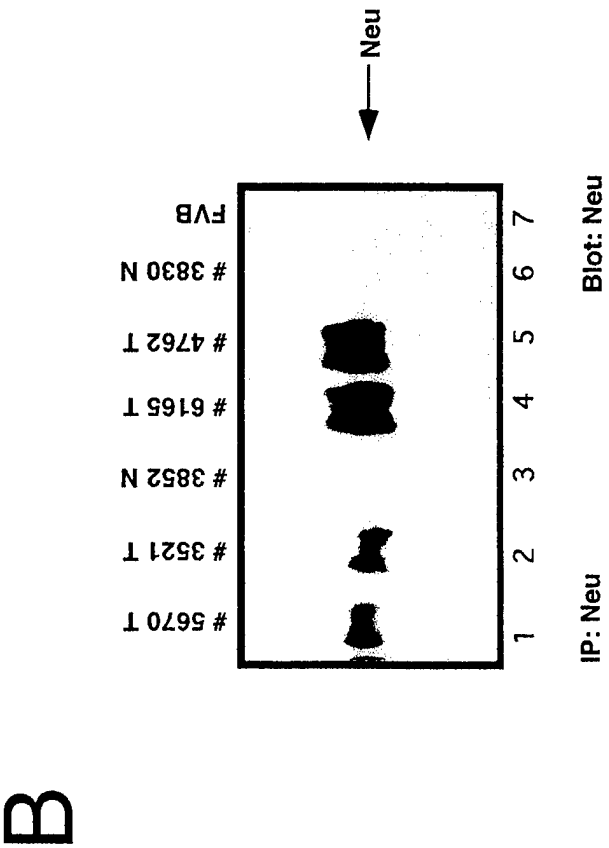
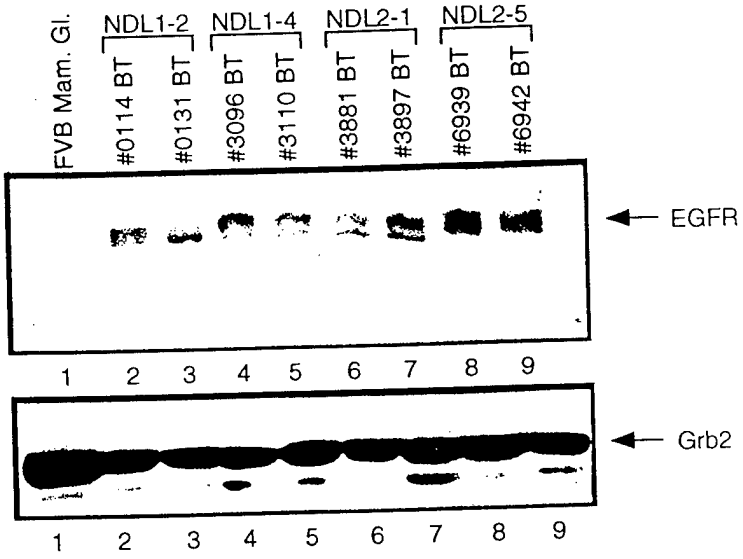
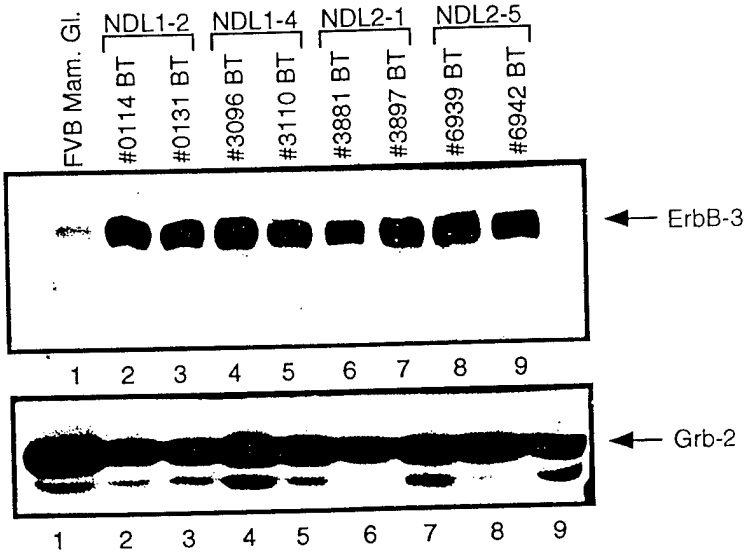

B
Neu
FVB
3830 N
4762 T
6165 T
3852 N
3521 T
5670 T
1 2 3 4 5 6 7
IP: Neu
Blot: Neu

FIG. 14. The expression of endogenous ErbB-3 receptors is significantly increased, relative to that of the EGFR and ErbB-4, in tumors derived from both NDL1 and NDL2 transgenic animals. (A) Equivalent amounts of total protein (50 μg) obtained from mammary tumor lysates (BT) were electrophoresed on an SDS-4 to 12% polyacrylamide gradient gel and transferred to a PVDF membrane (lanes 2 to 9). The membrane was cut, and the upper half of the blot was probed with EGFR-specific mouse monoclonal antibodies while the lower portion of the membrane was blotted with Grb2-specific rabbit polyclonal antisera. Similarly, immunoblots were performed for both ErbB-3 (B) and ErbB-4 (C) using the same tumor lysates. As in (A), Grb2 immunoblots were used to confirm that equivalent amounts of protein were present in each lane. Lysates from normal, non-transgenic mammary tissue (FVB Mam. Gl.) were included in each panel as a negative control (lane 1).

A


FVB Mam. Gl.
NDL1-2
NDL1-4
NDL2-1
NDL2-5
#0114 BT
#0131 BT
#3096 BT
#3110 BT
#3881 BT
#3897 BT
#6939 BT
#6942 BT
EGFR
1 2 3 4 5 6 7 8 9
Grb2
1 2 3 4 5 6 7 8 9

B


FVB Mam. Gl.
NDL1-2
NDL1-4
NDL2-1
NDL2-5
#0114 BT
#0131 BT
#3096 BT
#3110 BT
#3881 BT
#3897 BT
#6939 BT
#6942 BT
ErbB-3
1 2 3 4 5 6 7 8 9
Grb-2
1 2 3 4 5 6 7 8 9

C

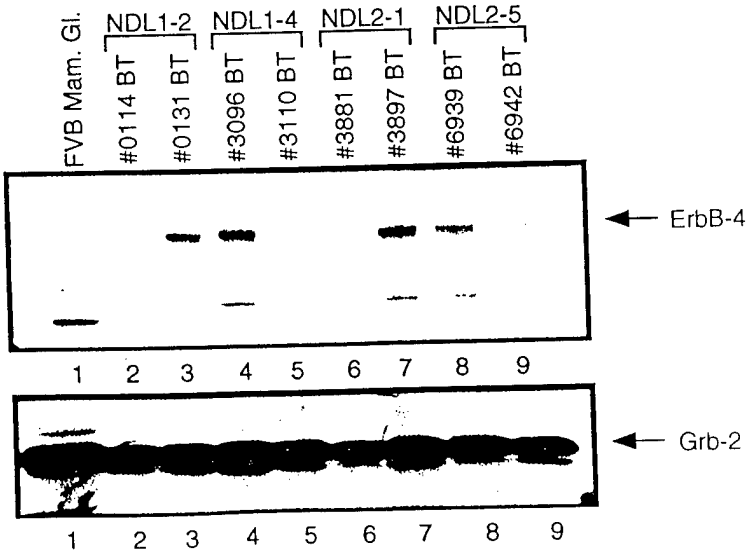
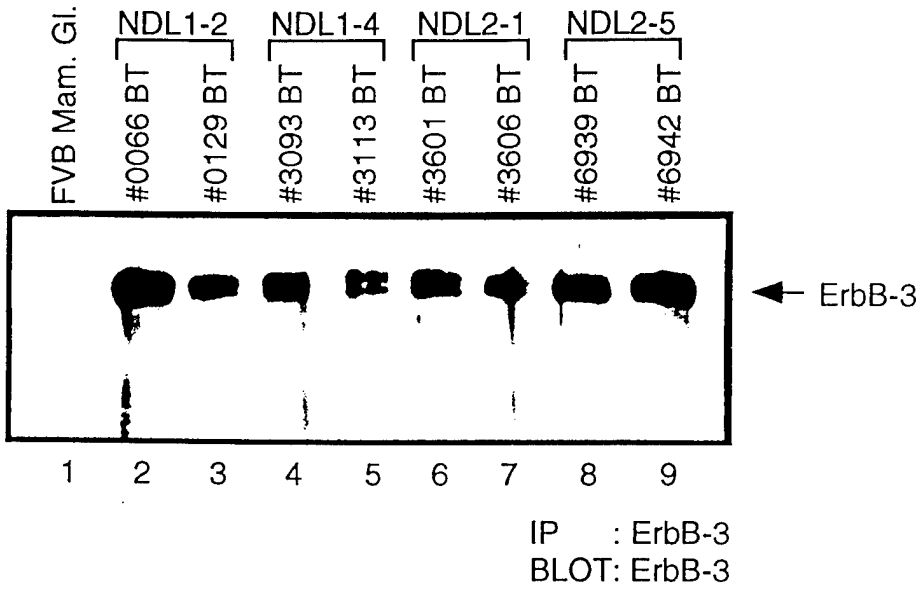

FVB Mam. Gl.
NDL1-2
NDL1-4
NDL2-1
NDL2-5
#0114 BT
#0131 BT
#3096 BT
#3110 BT
#3881 BT
#3897 BT
#6939 BT
#6942 BT
ErbB-4
1 2 3 4 5 6 7 8 9
Grb-2
1 2 3 4 5 6 7 8 9

FIG. 15. Endogenously expressed ErbB-3 is tyrosine phosphorylated. (A) ErbB-3 was immunoprecipitated (IP) from mammary tumor lysates (BT) derived from NDL transgenic animals (NDL1 and NDL2). One-third of the immunoprecipitate was electrophoresed on an SDS-9.0% polyacrylamide gel, transferred to a PVDF membrane, and subjected to immunoblot (BLOT) analyses with an ErbB-3 specific antibody (lanes 2 to 9). The position of ErbB-3 is indicated by the arrow. (B) The remaining two-thirds of the immunoprecipitate was subjected to immunoblot analyses with anti-phosphotyrosine (P-Tyr) specific antibodies, as described in (A). The position of tyrosine phosphorylated ErbB-3 is indicated by the arrow. A lysate from a non-transgenic mammary tissue (FVB Mam. Gl.) was included in the IP as a negative control (lane 1).

A


FVB Mam. Gl.
NDL1-2
NDL1-4
NDL2-1
NDL2-5
#0066 BT
#0129 BT
#3093 BT
#3113 BT
#3601 BT
#3606 BT
#6939 BT
#6942 BT
ErbB-3
1 2 3 4 5 6 7 8 9
IP : ErbB-3
BLOT: ErbB-3

B

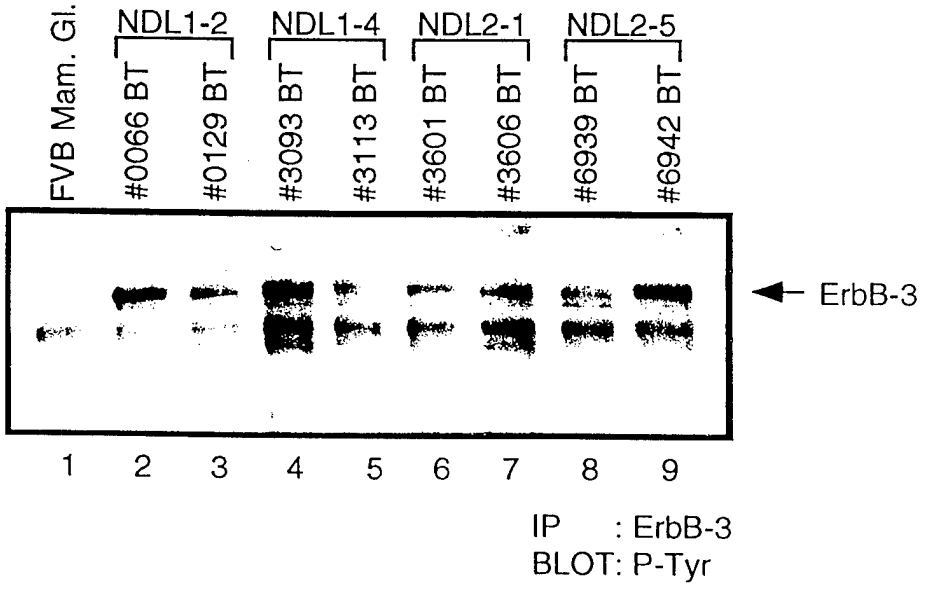

FVB Mam. Gl.
NDL1-2
NDL1-4
NDL2-1
NDL2-5
#0066 BT
#0129 BT
#3093 BT
#3113 BT
#3601 BT
#3606 BT
#6939 BT
#6942 BT
ErbB-3
1 2 3 4 5 6 7 8 9
IP : ErbB-3
BLOT: P-Tyr

FIG. 16. The levels of *erbB-3* transcript are not substantially increased in tumor versus adjacent mammary gland tissue. (A) Total RNA was isolated from mammary tumor tissue (BT) and adjacent mammary epithelium (Adj. M. Gl.) of three transgenic mouse strains (NDL1-2, NDL2-1 and N202). The 375 nt protected fragment corresponding to the *erbB-3 (B-3)* message is indicated. An antisense control probe, directed towards cytokeratin-8, was included to control for the epithelial content of the RNA sample. The 142 nucleotide protected fragment is indicated by *CK-8*. (B) The fold increase of *erbB-3* transcript in mammary tumors versus adjacent epithelium is indicated for each strain. Phosphoimager values for the *B-3* protected fragment were first divided by those obtained for the *CK-8* protected fragment to account for the epithelial content within each sample. These ratios were then used to determine the fold increase of *erbB-3* message in the mammary tumor versus adjacent epithelium. The number of samples analyzed are indicated (n).

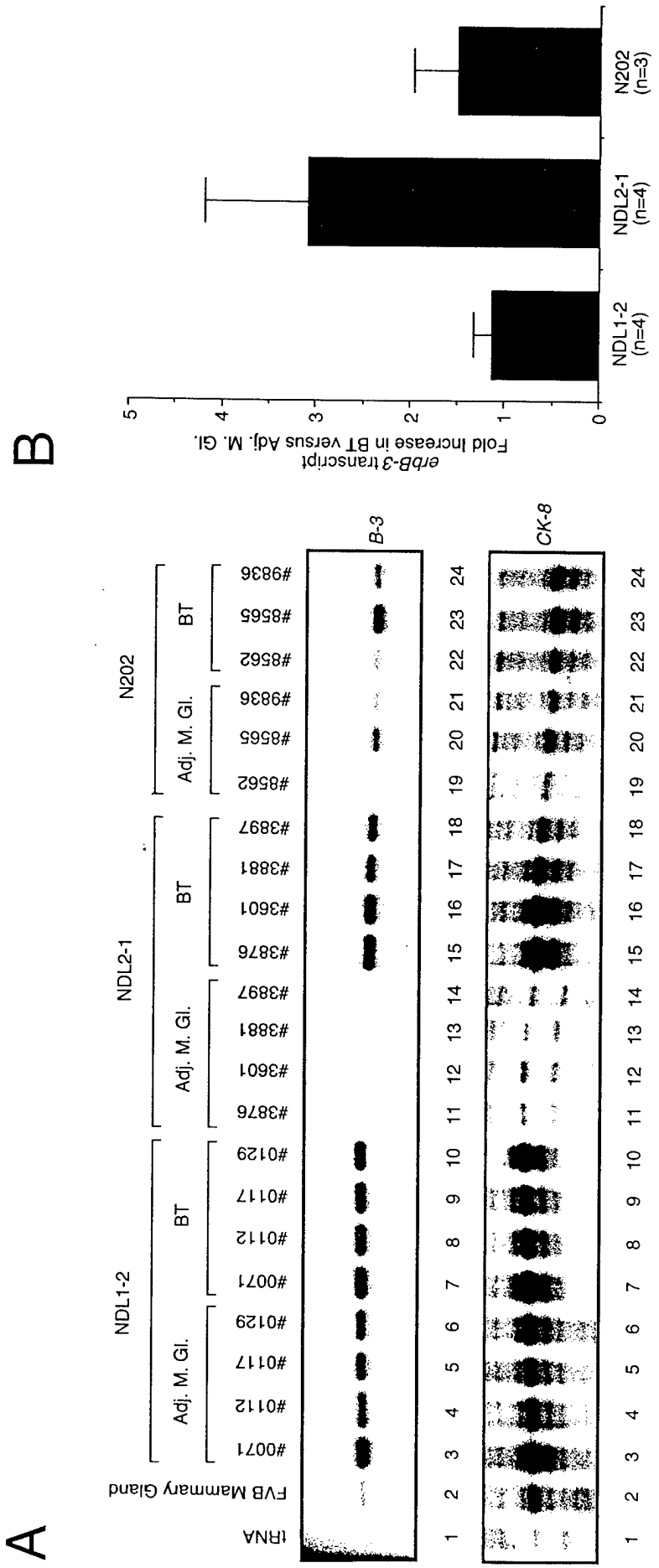

A
B
tRNA
FVB Mammary Gland
NDL1-2
Adj. M. Gl.
BT
NDL2-1
N202
#0071
#0112
#0117
#0129
#3876
#3601
#3881
#3897
#8562
#8565
#9836
B-3
CK-8
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
erbB-3 transcript
Fold Increase in BT versus Adj. M. Gl.
0 1 2 3 4 5
NDL1-2 (n=4)
NDL2-1 (n=4)
N202 (n=3)

FIG. 17. The levels of ErbB-3 protein are increased in tumor tissue versus adjacent mammary epithelium. Total protein (50 μg) was isolated from mammary tumor tissue (BT) and adjacent mammary epithelium (Adj. M. Gl.) of three transgenic mouse strains; NDL1-2 (A), NDL2-1 (B), and N202 (C). After separation of the protein lysates through SDS-9.0% polyacrylamide gels and transfer to a PVDF membrane, the blots were probed with antibodies specific to ErbB-3 (B-3) or Cytokeratin-8 (CK-8). I125 conjugated secondary antibodies were used for quantitatitve immunoblot analysis. (D) The fold increase of ErbB-3 protein in mammary tumors versus adjacent epithelium is indicated for each strain. Phosphoimager values for ErbB-3 were first divided by those obtained for Cytokeratin-8 in order to account for the epithelial content within each sample. These ratios were then used to determine the fold increase of ErbB-3 protein in mammary tumors versus adjacent epithelium. The number of samples analyzed are indicated (n).

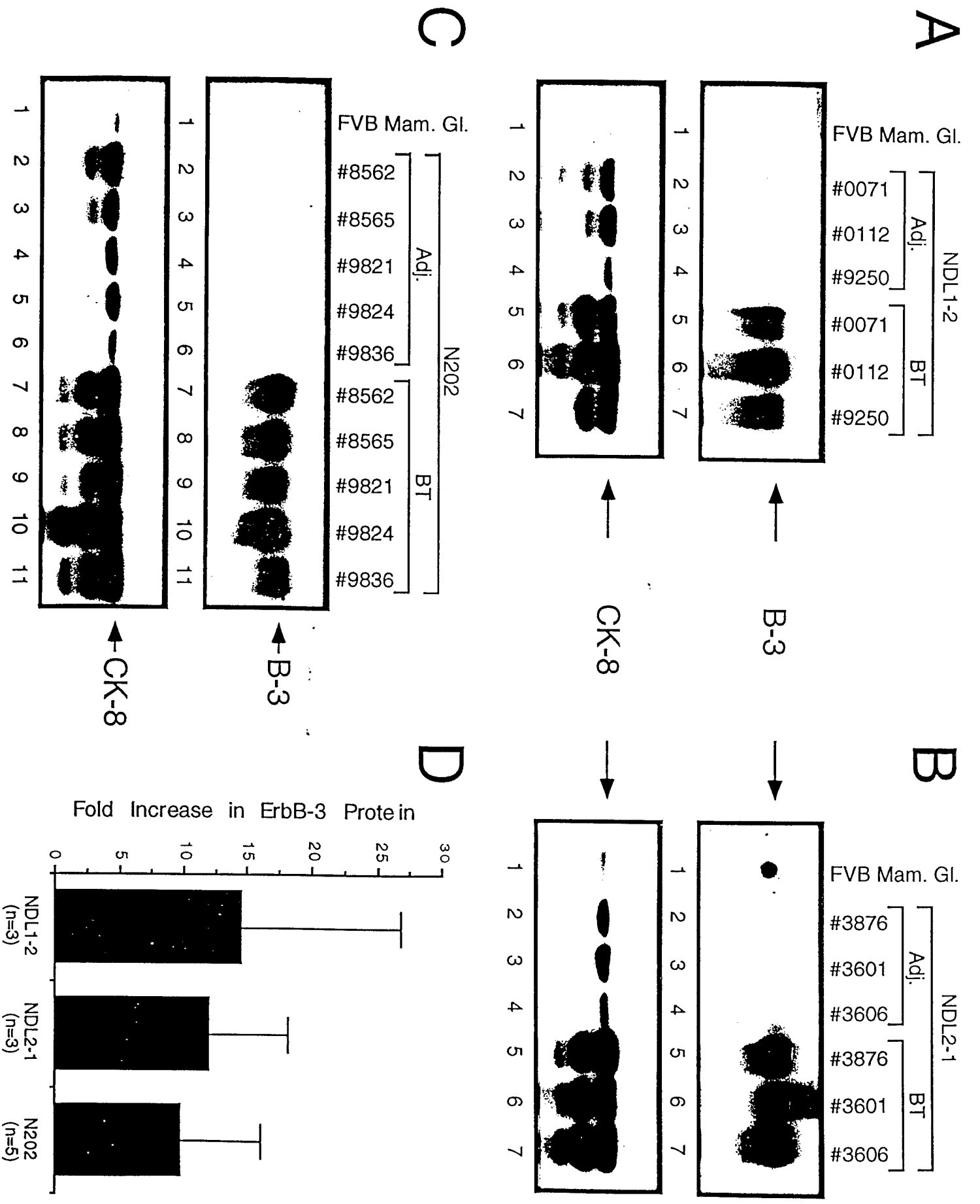
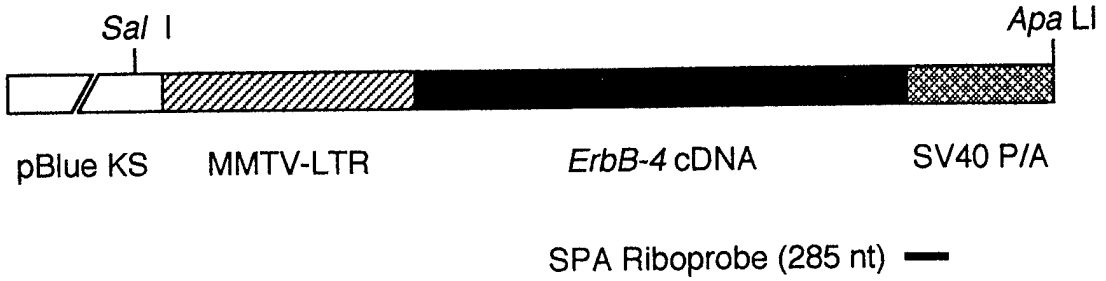

A
FVB Mam. Gl.
#0071
#0112
#9250
Adj.
#0071
#0112
#9250
BT
NDL1-2
B-3
CK-8
B
FVB Mam. Gl.
#3876
#3601
#3606
Adj.
#3876
#3601
#3606
BT
NDL2-1
C
FVB Mam. Gl.
#8562
#8565
#9821
#9824
#9836
Adj.
#8562
#8565
#9821
#9824
#9836
BT
N202
B-3
CK-8
D
Fold Increase in ErbB-3 Protein
0
5
10
15
20
25
30
NDL1-2 (n=3)
NDL2-1 (n=3)
N202 (n=5)

Figure 8 Structure of transgene and transgene expression in several transgenic lines. (A) Diagrammatic representation of the MMTV/erbB-4 transgene. The unshaded region represents Bluescript vector sequences, the striped region indicates the mouse mammary tumor virus long terminal repeat sequences, the filled in area represents the murine erbB-4 cDNA, and the grey region indicates transcriptional processing sequences derived from the SV40 early transcription unit. The 285 nt riboprobe utilized in the RNase protection shown in part B of this figure is indicated. (B) RNA transcripts corresponding to the MMTV/erbB-4 transgene in the mammary gland (females) and testes (male) from various transgenic lines. The first female in each set was a virgin animal while the second female in each set had given birth to one litter. The antisense riboprobe protects a 285 nt fragment indicated by SPA and an arrow. Also shown is an internal control riboprobe directed against the phosphoglycerate kinase gene. The PGK-1 riboprobe protects a fragment of 124 nt and is marked by PGK-1 and an arrow.

A


Sal I
Apa LI
pBlue KS
MMTV-LTR
ErbB-4 cDNA
SV40 P/A
SPA Riboprobe (285 nt)

B

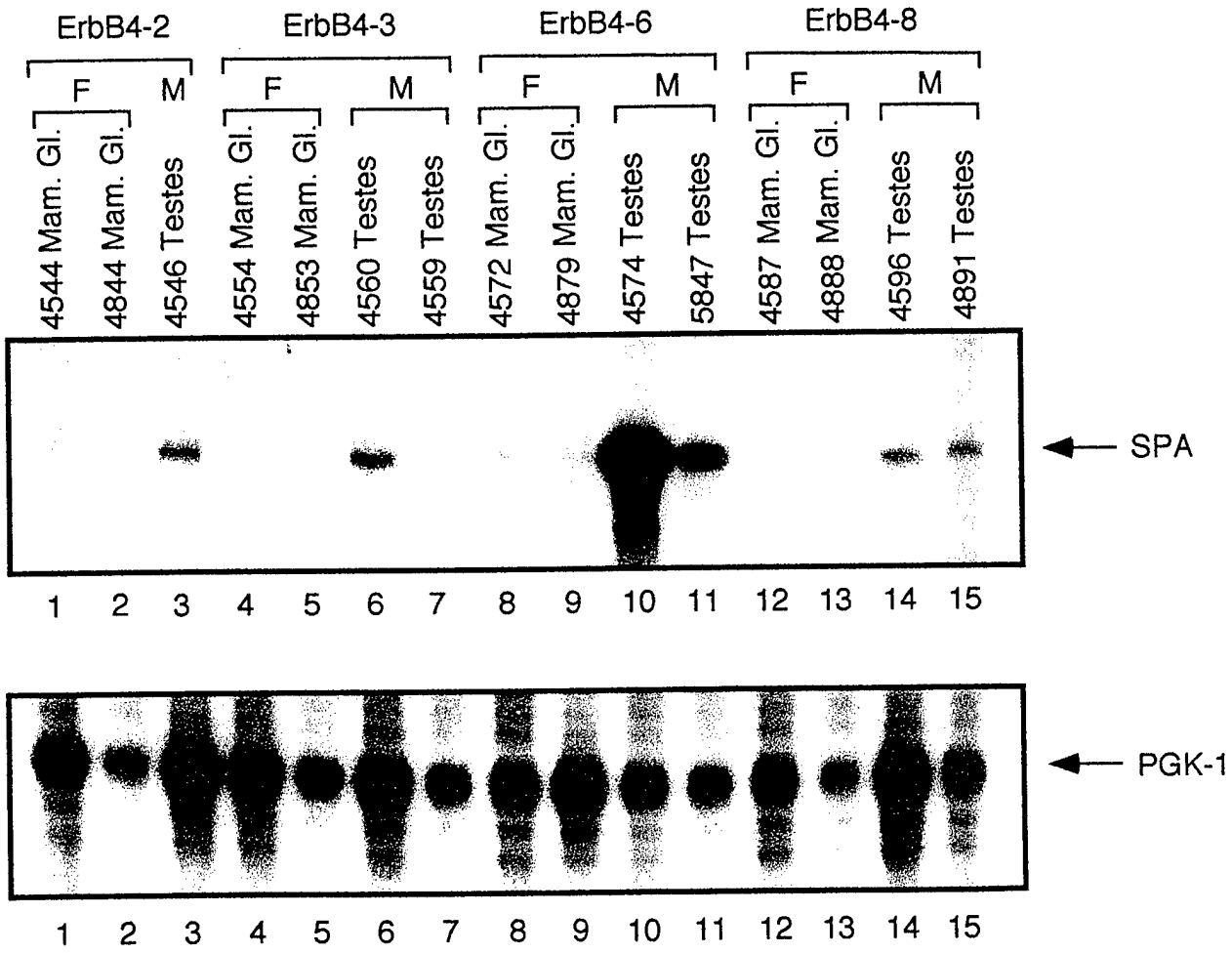
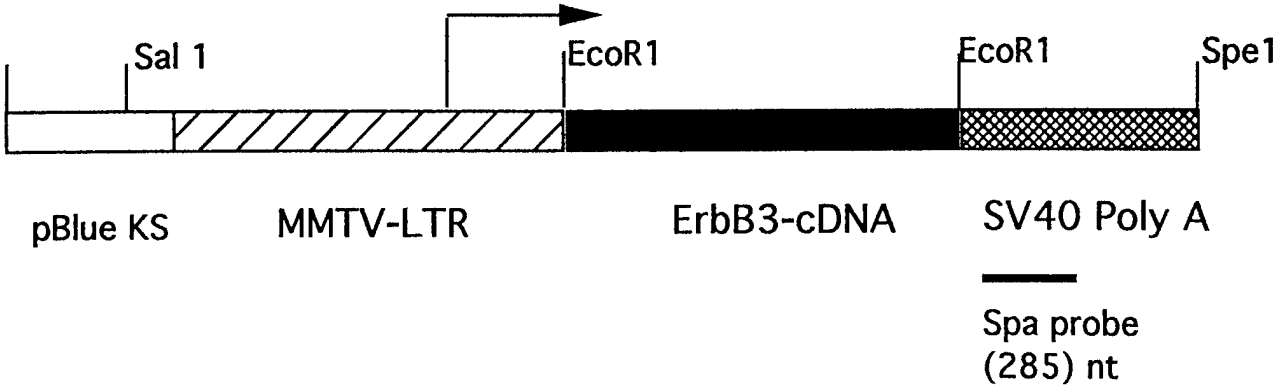

ErbB4-2
ErbB4-3
ErbB4-6
ErbB4-8
F
M
4544 Mam. Gl.
4844 Mam. Gl.
4546 Testes
4554 Mam. Gl.
4853 Mam. Gl.
4560 Testes
4559 Testes
4572 Mam. Gl.
4879 Mam. Gl.
4574 Testes
5847 Testes
4587 Mam. Gl.
4888 Mam. Gl.
4596 Testes
4891 Testes
SPA
PGK-1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

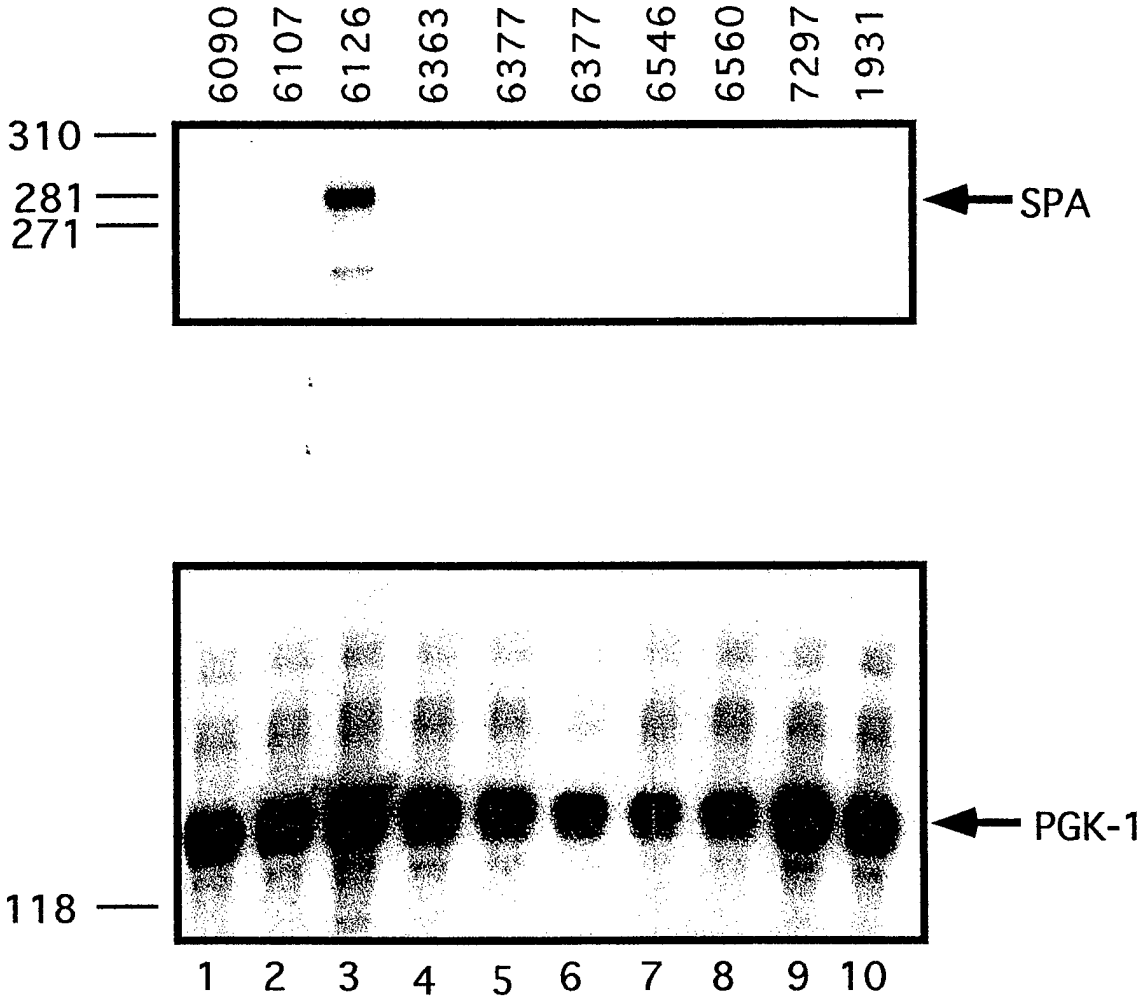
FIG. 19. Structure of erbB3 transgene and expression of erbB3 transgene in lactating mammary gland of various founder lines

(A) Transgene structure.The unshaded region indicates vector sequences (pBluescript), the striped region represents the MMTV-LTR, filled regions correspond to the erbB3 cDNA and the dotted region indicated polyadenylation signals derived from the SV40 early transcription unit. The Spa (SV40polyadenylation riboprobe used for the RNase protection analysis presented in (B) is also indicated. (B) RNA transcripts corresponding to the MMTV-erbB3 transgene in lactating mammary gland of various founder lines. The fragment protected by the antisense riboprobe is marked by SPA and an arrow. A riboprobe directed against the mouse phosphoglycerate kinase gene was also included in the hybridizations to control for equal loading of RNA and is marked by PGK-1 and an arrow. Migration of DNA markers is indicated (nucleotides) on the left side in panel (B).

A


Sal 1
EcoR1
EcoR1
Spe1
pBlue KS
MMTV-LTR
ErbB3-cDNA
SV40 Poly A
Spa probe
(285) nt

B


6090
6107
6126
6363
6377
6377
6546
6560
7297
1931
310
281
271
SPA
PGK-1
118
1 2 3 4 5 6 7 8 9 10

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Synergistic Interaction of the Neu Proto-Oncogene Product and Transforming Growth Factor α in the Mammary Epithelium of Transgenic Mice

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Transgenic mice expressing either the *neu* proto-oncogene or transforming growth factor (TGF-α) in the mammary epithelium develop spontaneous focal mammary tumors that occur after a long latency. Since the epidermal growth factor receptor (EGFR) and Neu are capable of forming heterodimers that are responsive to EGFR ligands such as TGF-α, we examined whether coexpression of TGF-α and Neu in mammary epithelium could cooperate to accelerate the onset of mammary tumors. To test this hypothesis, we interbred separate transgenic strains harboring either a mouse mammary tumor virus/TGF-α or a mouse mammary tumor virus/*neu* transgene to generate bitransgenic mice that coexpress TGF-α and *neu* in the mammary epithelium. Female mice coexpressing TGF-α and *neu* developed multifocal mammary tumors which arose after a significantly shorter latency period than either parental strain alone. The development of these mammary tumors was correlated with the tyrosine phosphorylation of Neu and the recruitment of c-Src to the Neu complex. Immunoprecipitation and immunoblot analyses with EGFR- and Neu-specific antisera, however, failed to detect physical complexes of these two receptors. Taken together, these observations suggest that Neu and TGF-α cooperate in mammary tumorigenesis through a mechanism involving Neu and EGFR transactivation.

The epidermal growth factor receptor (EGFR) family comprises four closely related type 1 receptor tyrosine kinases (RTKs) (EGFR, Neu [erbB-2, HER2], erbB-3 [HER3], and erbB-4 [HER4]) that are receptors for a variety of mitogenic growth factors (36). Enhanced expression of the EGFR family has been implicated in the genesis of human breast cancers. For example, amplification and consequent overexpression of *neu* have been observed in a significant proportion of human breast cancers and appear to be inversely correlated with patient survival (9, 12, 25, 32, 33, 37). More recently, overexpression of the other members of the EGFR family, including EGFR, erbB-3, and erbB-4, has also been implicated in the pathogenesis of human breast cancer (15, 16, 26, 27).

The activity of these EGFR family members can also be affected by expression of a variety of specific ligands for these RTKs. For example, Neu is a substrate of the activated EGFR following stimulation of cells with EGF or transforming growth factor α (TGF-α) (1, 8, 13, 35). Although Neu shares homology with the EGFR, Neu does not bind these EGF ligands. Rather, the observed tyrosine phosphorylation of Neu by the EGFR is thought to be mediated by heterodimerization and/or transactivation between Neu and EGFR, resulting in a high-affinity receptor for these EGFR ligands (8, 39). Consistent with these observations, coexpression of Neu and EGFR results in efficient transformation of fibroblasts in vitro (14). Moreover, elevated expression of both Neu and EGFR can be detected in primary human breast cancers (16). Taken together, these observations suggest that these two closely related RTKs may collaborate in mammary tumorigenesis.

Direct evidence of the involvement of EGFR family members in the induction of mammary tumors derives from observations made with transgenic mice expressing *neu* in the mammary epithelium (4, 10, 21). High-level expression of a constitutively active form of *neu* bearing a point mutation in the transmembrane domain (3) resulted in the development of nonstochastic, multifocal mammary tumors that affected every female carrier (21). In contrast, expression of the wild-type *neu* proto-oncogene in the mammary epithelium of transgenic mice resulted in the focal development of mammary tumors that arose after long latency (10). Interestingly, induction of mammary tumors in wild-type *neu* transgenic mice correlated with the frequent occurrence of activating mutations in the *neu* transgene (31). Thus, activation of the Neu RTK appears to be a pivotal step in the induction of mammary tumors in these mice.

Additional evidence implicating the EGFR family in mammary tumorigenesis derives from observations made with transgenic strains expressing an EGFR-specific ligand, TGF-α, in the mammary epithelium. Mammary gland-targeted expression of TGF-α in various transgenic strains results in the development of mammary epithelial hyperplasias that progress to focal mammary tumors after a long latency, as in wild-type *neu* transgenic mice (11, 18, 29). In mouse mammary tumor virus (MMTV)/TGF-α transgenic mice, increased expression of EGFR was observed in mammary tumors compared with adjacent, histologically normal tissue (18).

Given the potential of Neu and TGF-α (along with enhanced EGFR expression) to cooperate through a mechanism

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of receptor transactivation, we were interested in determining whether coexpression of *neu* and TGF-α could accelerate the induction of mammary tumors in vivo. To accomplish this, separate strains of transgenic mice carrying either an MMTV/*neu* or an MMTV/TGF-α transgene were interbred to generate dual carriers that coexpressed both *neu* and TGF-α in the mammary epithelium. The bigenic animals developed multifocal mammary tumors within a significantly shorter latency period than either parental strain alone. The induction of mammary tumors in these strains was further correlated to tyrosine phosphorylation of Neu and the recruitment of the c-Src tyrosine kinase to this complex, even in the absence of *neu* mutations. Cross-linking studies and reciprocal EGFR and Neu immunoprecipitations, however, did not demonstrate a physical association between EGFR and Neu in bigenic mice. Although these negative data do not disprove a transient physical association between these two receptors, the evidence favors receptor transactivation as the mechanism responsible for the observed cooperativity between EGFR and Neu in mammary tumorigenesis. Taken together, these data argue that coexpression of TGF-α and *neu* can act synergistically to transform the mammary epithelium.

MATERIALS AND METHODS

DNA constructs and generation of transgenic mice. The plasmid used to generate the antisense *neu* riboprobe was constructed by inserting an *SmaI-XbaI* fragment (nucleotides 1684 to 2332) into pSL301 (Invitrogen) (31). The phosphoglycerate kinase 1 (PGK-1) internal control plasmid was obtained from M. Rudnicki and was generated by inserting an *AccI-PstI* fragment (nucleotides 939 to 1633 of the PGK-1 cDNA) (20) into the *PstI* site of pSP64 (Promega). The TGF-α riboprobe was constructed by inserting the 632-bp *NcoI* fragment into the corresponding site in PSL301. The generation and characterization of both MMTV/wild-type *neu* and MMTV/TGF-α mice have been described previously (10, 18).

RNase protection assays. Total RNA was isolated from tissues by guanidinium thiocynate extraction, followed by CsCl gradient fractionation (5). The RNA yield was determined, after resuspension in sterile H_2O, by measuring the UV A_{260}. To generate the antisense *neu* probe, the template plasmids described above were linearized with *SmaI* and then subjected to an in vitro transcription reaction with T7 RNA polymerase (19). The PGK-1 internal control probe was produced by digesting the template plasmid with *Eco*NI and transcribing the product with SP6 RNA polymerase. The TGF-α riboprobe was generated by cleavage of the template plasmid with *XhoI*, followed by in vitro transcription with the T3 RNA polymerase. The RNase protection assays were performed by hybridizing the above-mentioned probes to 20 μg of total RNA as previously described (19). The protected fragments were separated on a sodium dodecyl sulfate (SDS)–6% polyacrylamide gel and subjected to autoradiography.

Immunoprecipitation and immunoblotting. Tissue samples were ground to powder under liquid nitrogen and lysed for 20 min on ice in TNE lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml). The lysates were cleared by centrifugation at 12,000 × *g* for 10 min at 4°C. Immunoprecipitations were performed by incubating 2.0 mg of the protein lysate with either 300 ng of anti-Neu monoclonal antibody 7.16.4 (6) or 300 ng of an anti-EGFR antibody (Transduction Laboratories catalog no. E12020) for 30 min at 4°C. Following incubation with protein G-Sepharose beads (Pharmacia) on a rotating platform at 4°C for 30 min, the precipitates were washed four times with TNE. The Neu and EGFR immunoprecipitates were resuspended in SDS-gel loading buffer, and the proteins were resolved on an SDS–9% polyacrylamide gel. The proteins were transferred onto a polyvinylidene difluoride membrane (Millipore) with an immunoblot transfer apparatus (Bio-Rad). Following overnight incubation in 3% bovine serum albumin (Sigma) in Tris-buffered saline (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM KCl), the membrane was probed for 2 h with antiphosphotyrosine antibodies (1:500; Upstate Biotechnology, Inc.) in bovine serum albumin in Tris-buffered saline. After being washed in Tris-buffered saline–0.05% Tween 20, the blots were incubated in 3% milk in Tris-buffered saline for 1 h. The membrane was incubated with goat anti-mouse immunoglobulin G, and the proteins were visualized by the enhanced-chemiluminescence detection system (Amersham).

For studies demonstrating the in vivo association of Neu with c-Src, tumor lysates were prepared and cleared as described previously (24). Proteins were immunoprecipitated by incubating 1.0 to 2.0 mg of total cell lysate with 2 μg of anti-c-Src antibody 7D10 (Quality Biotech) for 3 h at 4°C and subsequently washed five times with lysis buffer. The samples were resolved on an SDS–8% polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane. The membrane was probed with anti-Neu antibody AB.3 (1:1,000; Oncogene Science) as previously described (24).

For studies examining receptor association, tumor membranes were prepared from 0.3- to 0.6-g tissue aliquots by homogenization in a detergent-free hypotonic buffer as described previously (2). Following 45 min of ultracentrifugation at 100,000 × *g* and 4°C, pellets were solubilized for 45 min in 3-[(3-cholamidopropyl)-dimethyl-amonio]-1-propanesulfonate (CHAPS) buffer (50 mM Tris [pH 8.0], 0.7% CHAPS, 50 mM NaCl, 1 mM sodium orthovandate, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml). Nonidet P-40-insoluble material was removed by centrifugation at 14,000 × *g* for 15 min. Equivalent amounts of membrane protein were immunoprecipitated with either Neu polyclonal antiserum 21N (30) or anti-EGFR polyclonal antiserum 986 (30) and Staph A cells (Calbiochem) for 1 to 2 h. After washes, both Neu and EGFR immunoprecipitates were subjected to immunoblot analyses with the 21N polyclonal antiserum or an anti-EGFR monoclonal antibody (Transduction Laboratories). For detection, horseradish peroxidase-linked sheep anti-rabbit or anti-mouse antibodies were utilized. Direct binding assays were performed primarily as described previously (23).

Histological evaluation. Complete autopsies were performed. Tissues were fixed in 4% paraformaldehyde, sectioned at 4 μm, routinely stained with hematoxylin and eosin, and examined as indicated in the legend to Fig. 4. Whole-mount analyses were performed as previously described (11).

RESULTS

Detection of TGF-α and *neu* transcripts in mammary epithelium of transgenic mice carrying both MMTV/*neu* and MMTV/TGF-α transgenes. To determine if coexpression of TGF-α and *neu* could cooperate in mammary tumorigenesis, separate strains of MMTV/*neu* and MMTV/TGF-α were interbred to generate offspring carrying both transgenes. The MMTV/TGF-α strain is derived from line 29 and originates from a C57BL × DBA genetic background (18), whereas the MMTV/*neu* strain is derived from the N#202 founder animal and is derived from an inbred FVB genetic background (10). Because TGF-α-expressing females are unable to nurse their young, the F_1 progeny from this cross were generated by crossing MMTV/TGF-α males with MMTV/*neu* females. Females derived from this cross were segregated into breeding and virgin female groups. Analyses of the multiparous animals derived from this cross revealed that dual carriers possessing both the TGF-α and *neu* transgenes were incapable of nursing their young, like TGF-α-expressing females. In addition to the apparent lactation defect, multiparous female transgenic mice bearing the TGF-α and *neu* transgenes exhibited uniform hypertrophy of the mammary glands (22).

To assess whether the mammary epithelium derived from the various genotypes expressed the appropriate transgenes, RNAs derived from the mammary tumors derived from *neu*/TGF-α or *neu*/+ virgin transgenic mice were subjected to RNase protection analyses with riboprobes specific for *neu* (Fig. 1A) or TGF-α (Fig. 1B). To ensure equal loading of RNA, a PGK-1 antisense probe (20) was also included in the hybridization reaction mixtures. Examination of RNA samples derived from the tumors of eight MMTV/*neu* female mice revealed increased levels of *neu* transcripts. Interestingly, several of these tumor samples demonstrated evidence of altered transcripts (Fig. 1A, lanes 9, 10, and 13 to 15). Indeed, previous studies have demonstrated that these altered transcripts invariably encode in-frame deletions in the extracellular domain of Neu which result in its oncogenic activation (31). As expected, elevated *neu* transcript levels were observed in mammary tumors derived from bitransgenic animals harboring both transgenes. Significantly, the mammary tumor RNA samples obtained from seven dual transgene carriers did not exhibit evidence of altered transcripts that were observed in the *neu*-induced tumors (Fig. 1A, lanes 1 to 7).

An identical RNase protection analysis was performed on these RNA samples to assess the levels of TGF-α (Fig. 1B). These analyses revealed that TGF-α could be detected in

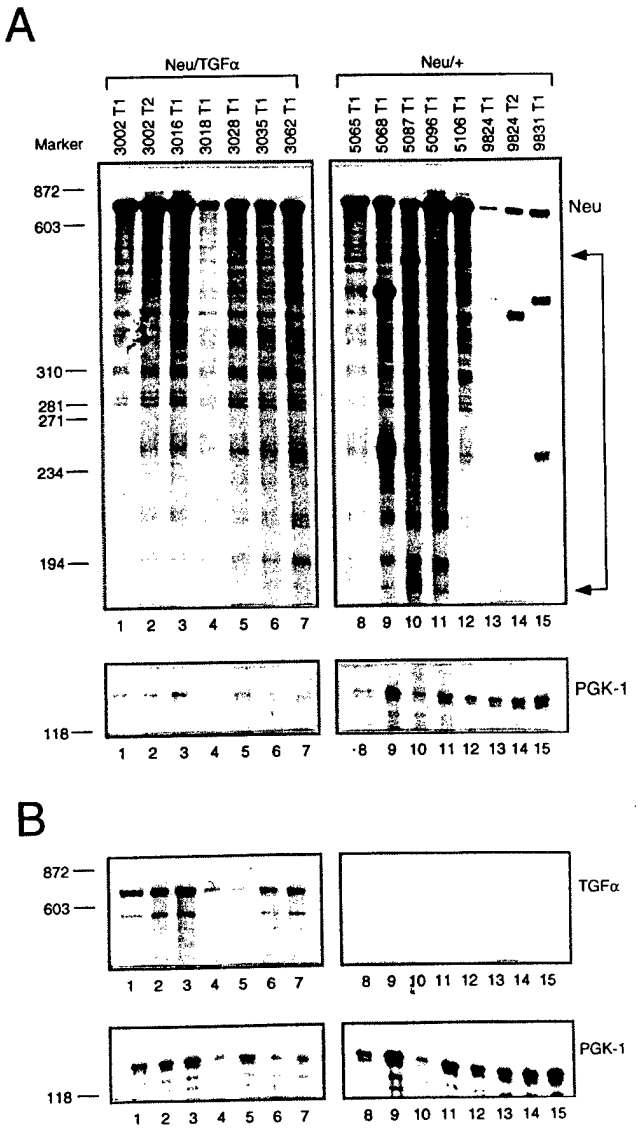

FIG. 1. Expression of Neu and TGF-α transgenes in mammary tissue of transgenic mice. (A) Neu transgene expression in mammary tissues of mice carrying the MMTV/*neu* transgene (*neu*/+) and both transgenes (Neu/TGF-α). RNA samples derived from tumor tissue (T) were subjected to RNase protection analyses. The protected wild-type *neu* transcript is 640 nucleotides long. Protected fragments corresponding to the altered *neu* transcript are indicated by arrows. Tumor RNA samples were derived from virgin female animals. An antisense riboprobe directed against the mouse PGK-1 gene was used to control for equal loading of RNA on the gel. The PGK-1 probe protects a 124-nucleotide fragment, as indicated in the lower panels. (B) The identical RNA tissue samples were hybridized with an antisense probe directed against the mouse TGF-α gene. The TGF-α antisense probe protects a 632-nucleotide fragment. The PGK-1 probe protects a 124-nucleotide fragment, as indicated in the lower panels. The numbers on the left are molecular sizes in nucleotides.

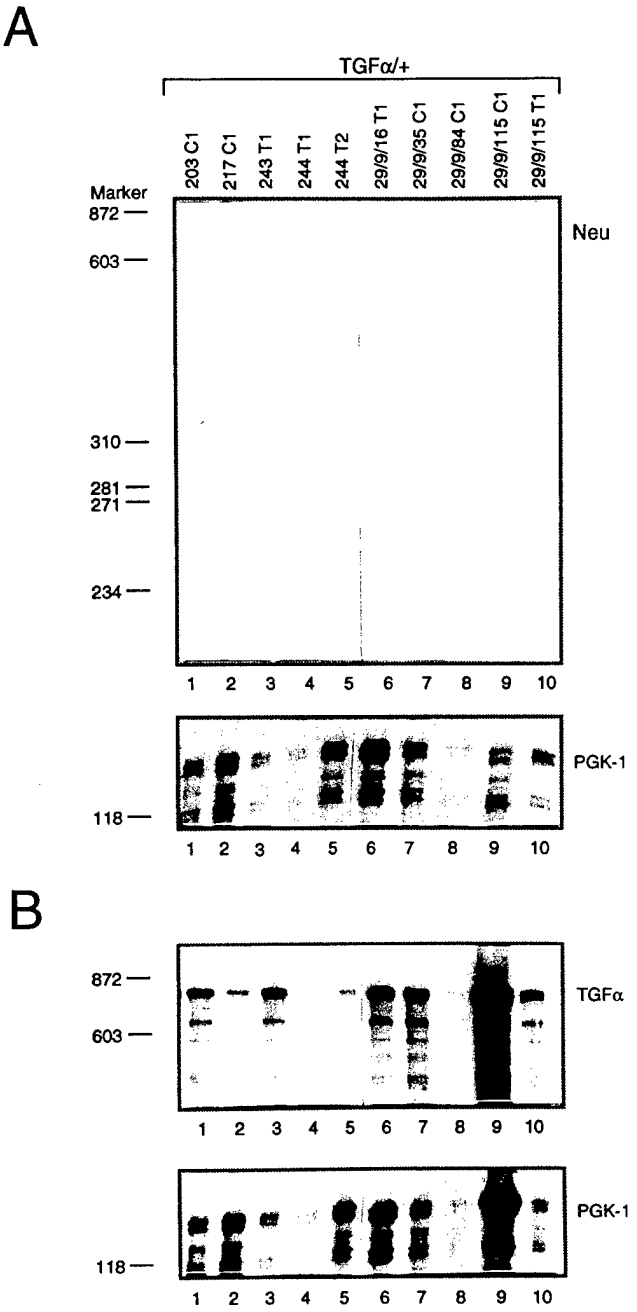

FIG. 2. Expression of Neu and TGF-α transcripts in tumors and hyperplasias derived from MMTV/TGF-α transgenic mice. (A) Endogenous Neu expression in mammary tissues of mice expressing the TGF-α transgene (TGF-α/+). Tumor (T) and cystic hyperplastic (C) tissue RNA samples from virgin female TGF-α carriers were subjected to RNase protection analyses with a *neu* riboprobe. The protected *neu* transcript is 640 nucleotides long. An antisense riboprobe directed against the mouse PGK-1 gene was used to control for equal loading of RNA on the gel. The PGK-1 probe protects a 124-nucleotide fragment, as indicated in the lower panel. (B) RNA tissue samples identical to those in panel A were probed with an antisense probe directed against the mouse TGF-α gene. The TGF-α antisense probe protects a 632-nucleotide fragment. The numbers on the left are molecular sizes in nucleotides.

mammary tumor RNA samples derived from both MMTV/TGF-α mice (Fig. 2B, lanes 1 to 10) and mice carrying both the *neu* and TGF-α transgenes (Fig. 1B, lanes 1 to 7). In contrast, no detectable transcripts corresponding to the TGF-α transgene were detected in mammary tumor RNA samples from transgenic mice carrying the *neu* transgene alone (Fig. 1B, lanes 8 to 15). Analyses of a representative sample of tumors and cystic hyperplasias derived from virgin females carrying the MMTV/TGF-α transgene alone with the identical riboprobes revealed no evidence of expression of *neu* (Fig. 2A, lanes 1 to 10); however, high TGF-α transcript levels were detected in these tissues (Fig. 2B, lanes 1 to 10). Seven MMTV/TGF-α tumor samples and five cysts were analyzed by

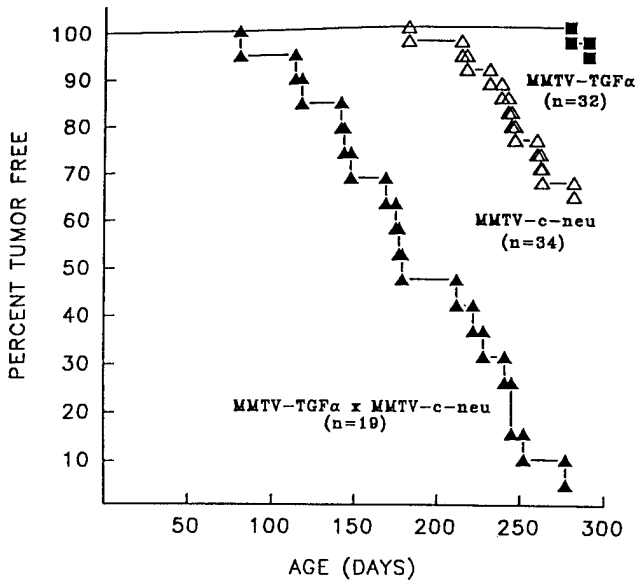

FIG. 3. Kinetics of tumor occurrence in monogenic and bigenic animals harboring the MMTV/TGF-α and MMTV/*neu* transgenes. Comparison of the kinetics of tumor formation between virgin female carriers bearing the MMTV/TGF-α, MMTV/*neu*, and both transgenes. The numbers of mice examined are indicated.

RNase protection. Taken together, these observations indicate that the dual carriers coexpress elevated levels of TGF-α and *neu* in the mammary epithelium.

Coexpression of TGF-α and *neu* in mammary epithelium results in rapid generation of multifocal mammary tumors. To test whether *neu* and TGF-α could collaborate in mammary tumorigenesis, virgin female mice carrying the *neu*, TGF-α, or both transgenes were monitored for the physical appearance of mammary tumors by palpation weekly. As shown in Fig. 3, the appearance of mammary tumors in transgenic mice expressing TGF-α or *neu* alone occurred only after long latency. Only 6% of the MMTV/TGF-α and 35% of the MMTV/*neu* mice developed palpable mammary tumors by 250 days (Fig. 3). In contrast, 95% of the bigenic mice developed palpable tumors by this time (Fig. 3), and 50% of these dual carriers had tumors by 175 days, at which time neither the TGF-α- nor the *neu*-expressing mice exhibited tumors (Fig. 3). In addition to the accelerated onset of mammary tumors, the mammary tumors in the bigenic mice developed were multifocal and encompassed the entire mammary epithelium, whereas transgenic mice expressing either TGF-α or *neu* alone developed mammary tumors that were generally focal in origin.

To explore the phenotypic differences between the various transgenic mice, mammary fat pads derived from age-matched virgin female mice were subjected to whole-mount analyses (Fig. 4). The results showed that mice carrying the *neu* transgene possessed mammary trees indistinguishable from those of virgin FVB female mice (Fig. 4B); however, the mammary trees of either the TGF-α or the *neu*/TGF-α mice were grossly abnormal (Fig. 4C and D). In fact, both the TGF-α and *neu*/TGF-α mice displayed extensive lobuloalveolar development resembling that of a normal FVB lactating female mouse (Fig. 4A). Careful examination of whole-mount preparations derived from TGF-α and *neu*/TGF-α mice revealed clear differences between the two. The alveoli present in the *neu*/TGF-α mice contained a denser cell lining in the walls (Fig. 4D) than did the large, cystically dilated alveoli found in the TGF-α mice (Fig. 4C). Consistent with these whole-mount findings, histological examination of these mammary hyperplasias derived from the MMTV/TGF-α mice had extensive lobular development (Fig. 5C) which resembled that of a lactating nontransgenic animal (Fig. 5A), except that the alveoli were irregular and dilated (Fig. 5C). Further, the interstitial stroma was edematous and had a modest increase in mononuclear and polymorphonuclear leukocytes. The mammary gland of the nulliparous female had minimal lobular development and no evidence of a stromal response (Fig. 5B). In contrast, the mammary gland of the bitransgenic animal frequently showed epithelial hyperplasia and dysplasia along with stromal inflammation (Fig. 5D). Therefore, the presence of inflammatory stroma tissue in the mammary gland is closely associated with expression of the MMTV/TGF-α transgene. The mammary tumors arising from the monogenic animals were typical of those previously reported: TGF-α-expressing animals demonstrated tubular adenocarcinomas, whereas *neu*-expressing mice typically developed nodular tumors (10, 18). The tumors arising in the bigenic *neu*/TGF-α animals were interesting in that they were either nodular (Fig. 6A) or tubular (Fig. 6B) but did not demonstrate a mixed or different morphological pattern; however, both categories of tumors coexpressed *neu* and TGF-α (Fig. 1). Together with the histological observations, these findings suggest that coexpression of *neu* and TGF-α is associated with induction of widespread morphological abnormalities in the mammary gland.

Constitutive tyrosine phosphorylation of Neu in mammary tissues coexpressing TGF-α and *neu*. The results above strongly suggest that coexpressed TGF-α and *neu* are highly synergistic in their capacity to transform the mammary epithelium. A probable biochemical explanation for this observation is that TGF-α activates Neu-associated tyrosine kinase activity by transphosphorylation through the activated EGFR, especially in light of the lack of activating mutations of *neu* in the bigenic tumors. To explore this possibility, protein extracts obtained from the mammary glands of MMTV/*neu* mice, MMTV/TGF-α mice, and dual carriers were subjected to immunoprecipitation analyses with either EGFR- or Neu-specific antiserum and immunoblotted with phosphotyrosine-specific antiserum (Fig. 7B and D, respectively). To control for the amount of protein, the same immunoprecipitates were also immunoblotted with either EGFR- or Neu-specific antiserum (Fig. 7A and C, respectively). Low but detectable quantities of EGFR protein were found in tumor samples from transgenic animals expressing either Neu or TGF-α alone (Fig. 7A, lanes 1 to 6); tumors coexpressing TGF-α and Neu expressed variable levels of EGFR (Fig. 7A, lanes 7 to 10). The levels of tyrosine-phosphorylated EGFR in these tumor samples directly correlated with the results of the immunoblot analyses (Fig. 7B). In contrast to the variable expression of EGFR, extremely high levels of tyrosine-phosphorylated Neu were detected in mammary tumors induced by the *neu* transgene alone or by both transgenes (Fig. 7D, lanes 1 to 3 and 7 to 10), which correlated with the total levels of Neu protein immunoprecipitated (Fig. 7C, lanes 1 to 3 and 7 to 10). No detectable tyrosine-phosphorylated Neu was observed in mammary protein samples from mice expressing TGF-α alone (Fig. 7C and D, lanes 4 to 6).

To further explore the mechanism by which Neu was transactivated in mammary tumors coexpressing TGF-α and *neu*, protein lysates derived from *neu*/TGF-α-, *neu*-, or TGF-α-expressing mice were subjected to reciprocal immunoprecipitation and immunoblot analyses with antisera specific to EGFR and Neu (Fig. 8). Although immunoprecipitation of these protein lysates with either Neu- or EGFR-specific anti-

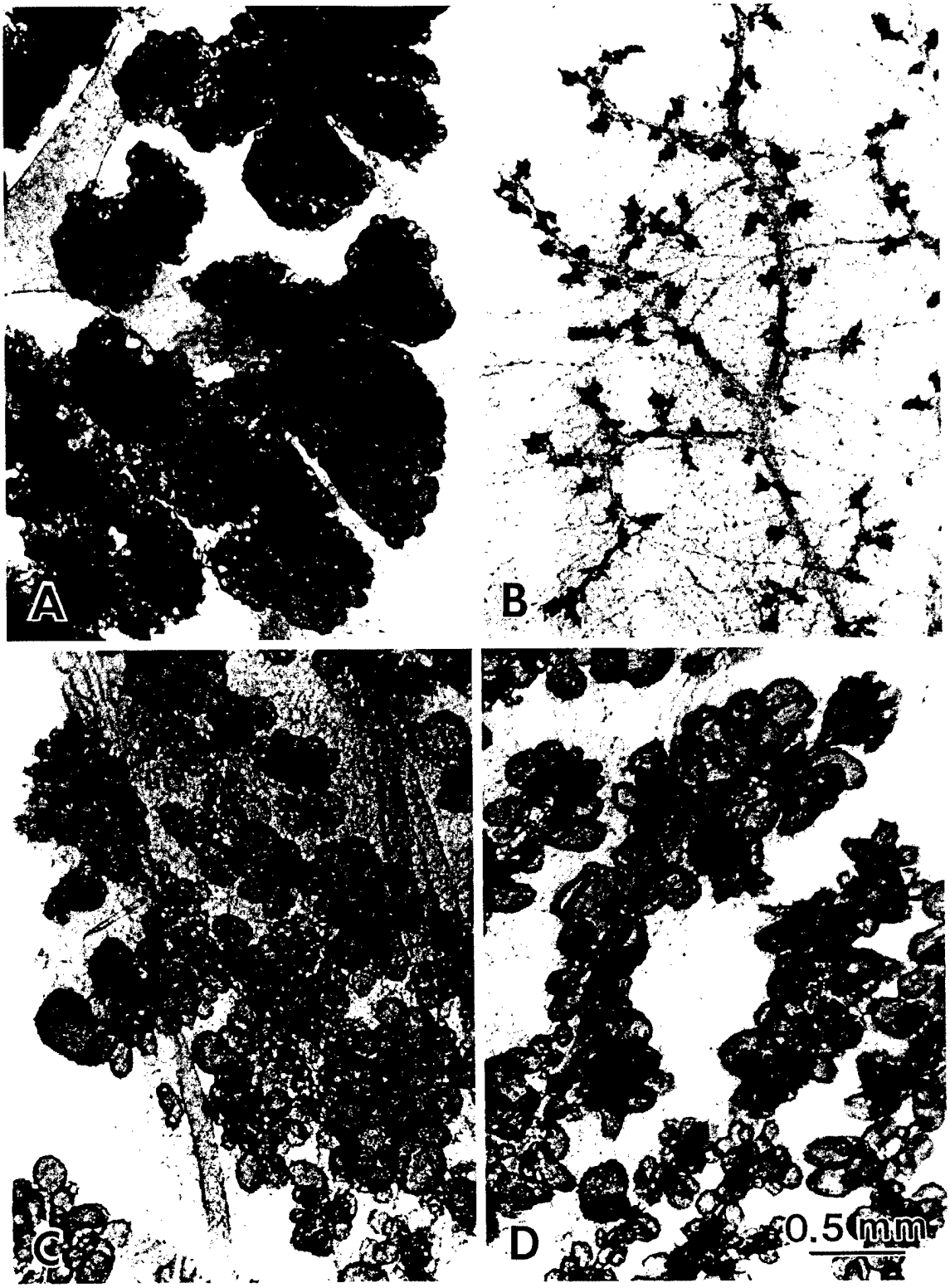

FIG. 4. Whole-mount analyses of mammary fat pads derived from monogenic and bigenic female mice. Shown are whole-mount preparations illustrating the appearance of mammary trees from a lactating FVB female (A), a virgin female with the *neu* transgene (note the numerous side buds which give the mammary tree a spiculated appearance) (B), a virgin female with the TGF-α transgene (note the well developed, cystically dilated alveoli) (C), and a virgin female with both the TGF-α and *neu* transgenes (note the larger cystic alveoli with darker walls, indicating a denser cell lining in the walls) (D). Compare these preparations with the comparable histologic preparations in Fig. 5. Magnification, ×31.5.

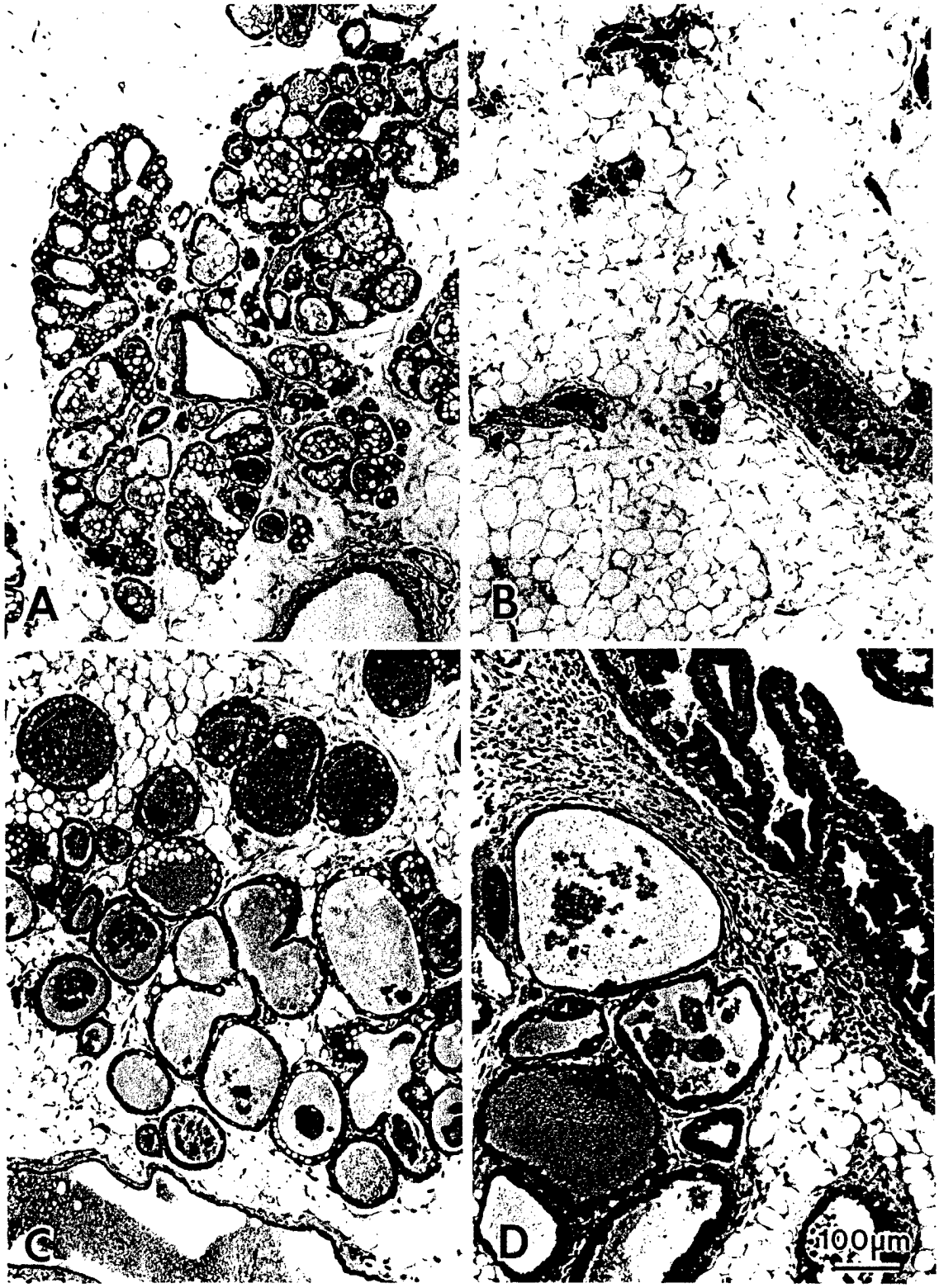

FIG. 5. Histopathology of mammary tissues derived from virgin monogenic and bigenic transgenic animals. (A) Normal FVB lactating female mouse showing lobuloalveolar development and milk production. (B) Transgenic *neu* virgin female mouse illustrating rudimentary mammary acinar development without significant luminal secretions. (C) Transgenic TGF-α virgin female mouse illustrating extensive alveolar development in comparison with a lactating mammary gland (A). Note that the alveoli are much more distended with secretory products than the FVB lactating tissue but contain fewer clear lipid vacuoles. (D) Transgenic *neu*/TGF-α virgin female mouse illustrating areas of alveolar development with papillary hyperplasia in the upper right corner. The virgin *neu*, TGF-α, and *neu*/TGF-α mice were age matched (139 days) and identical to those described in Fig. 4.

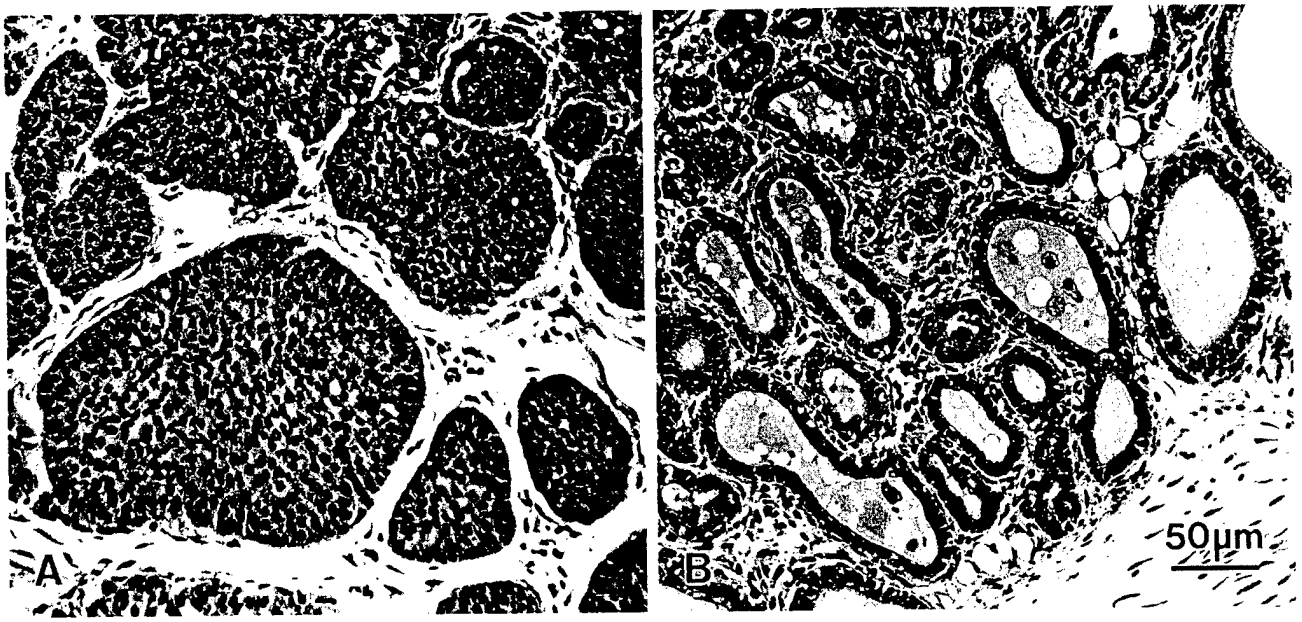

FIG. 6. Histology of the two types of mammary tumors observed in *neu*/TGF-α bigenic mice. (A) Nodular tumor typical of the Neu phenotype. (B) Tubular type typical of the TGF-α phenotype. Magnification, about ×147.

bodies, followed by immunoblot analyses with Neu-specific antiserum, revealed abundant Neu protein in the immunoprecipitates from the *neu*/TGF-α- and *neu*-expressing tumors, no detectable Neu was found in the EGFR immunoprecipitates (Fig. 8A). Conversely, immunoprecipitation with EGFR-specific antibodies, followed by immunoblot analyses with Neu-specific antiserum, failed to demonstrate the presence of EGFR in these complexes (Fig. 8B). Thus, under these experimental conditions, we could not detect a physical interaction between the EGFR and Neu in bigenic mice, and these findings indicate that transactivation of Neu by TGF-α does not involve the formation of stable Neu-EGFR heterodimers.

Induction of mammary tumors by *neu* and *neu*–TGF-α transgenes correlates with the capacity of Neu to associate with the c-Src tyrosine kinase in vitro and in vivo. One possible explanation for the observed synergy between Neu and TGF-α/EGFR is that these closely related type 1 receptors recruit distinct but complementary pathways. Indeed, we have previously demonstrated that activation of c-Src by the activated EGFR in fibroblasts is mediated by direct and specific association of c-Src with Neu (23). To test whether the c-Src signaling pathway was also recruited to Neu in *neu*/TGF-α-expressing tumors, Neu immunoprecipitates derived from *neu*, TGF-α, and *neu*/TGF-α tumor samples were resolved on an SDS-polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane, and probed with a radiolabeled glutathione *S*-transferase (GST) fusion protein containing the SH2 domain of c-Src (GSTag–c-Src–SH2) (Fig. 9A). Consistent with previous observations (23), the radiolabeled GSTag–c-Src–SH2 probe bound to the Neu immunoprecipitates derived from mammary tumors expressing Neu alone (Fig. 9A, lanes 1 to 3). By contrast, the radiolabeled fusion protein failed to bind the Neu immunoprecipitates derived from the TGF-α-induced tumors (Fig. 9A, lanes 4 to 6). An identical analysis of the Neu immunoprecipitates derived from tumors coexpressing both Neu and TGF-α revealed that they also bound strongly to the radiolabeled GSTag–c-Src–SH2 probe (Fig. 9A, lanes 7 to 10). The observed binding of the GSTag–c-Src–SH2 probe is likely specific to tyrosine-phosphorylated Neu, since previous studies have demonstrated that the radiolabeled GSTag–c-Src–SH2 probe cannot bind comparable levels of tyrosine-phosphorylated EGFR (23). Thus, Neu derived from *neu*/TGF-α tumors is capable of interacting with c-Src in a direct manner in vitro.

To confirm that c-Src could interact with Neu in vivo, the same protein lysates were immunoprecipitated with c-Src-specific antiserum and subjected to immunoblot analyses with Neu-specific antiserum (Fig. 9B). Consistent with the in vitro binding data, Neu protein was found in c-Src immunoprecipitates derived from tumors expressing *neu* alone or coexpressing both *neu* and TGF-α (Fig. 9B, lanes 1, 2, and 5 and 7) but was absent from tumors expressing TGF-α alone since the latter fail to express detectable levels of Neu (Fig. 9B, lanes 3 and 4). Taken together, these observations suggest that transactivation of nonmutated Neu by the activated EGFR results in recruitment of the c-Src signaling pathway.

DISCUSSION

Our results show that coexpression of *neu* and TGF-α in the mammary epithelium of transgenic mice results in the induction of multiple growth disturbances in the mammary epithelium, leading to tumor formation. We also present evidence that the occurrence of these growth disturbances correlates with the constitutive activation of the tyrosine kinase activity of Neu. These observations suggest that TGF-α and *neu* cooperate in mammary tumorigenesis, possibly through transactivation of Neu by the EGFR.

The phenotype exhibited by transgenic mice coexpressing TGF-α and *neu* provides important insight into the interaction of EGFR family members in mammary tumorigenesis. Virgin female mice coexpressing TGF-α and *neu* demonstrated dramatic and distinct mammary morphological differences in comparison with either parental strain (Fig. 4 and 5). In addition, dual transgene carriers developed mammary tumors with greater penetrance and shorter latency than either *neu* or TGF-α animals alone (Fig. 3). One of the most striking features of the mammary tumor tissue derived from bigenic animals is the hyperproliferation of the stromal tissue adjacent to

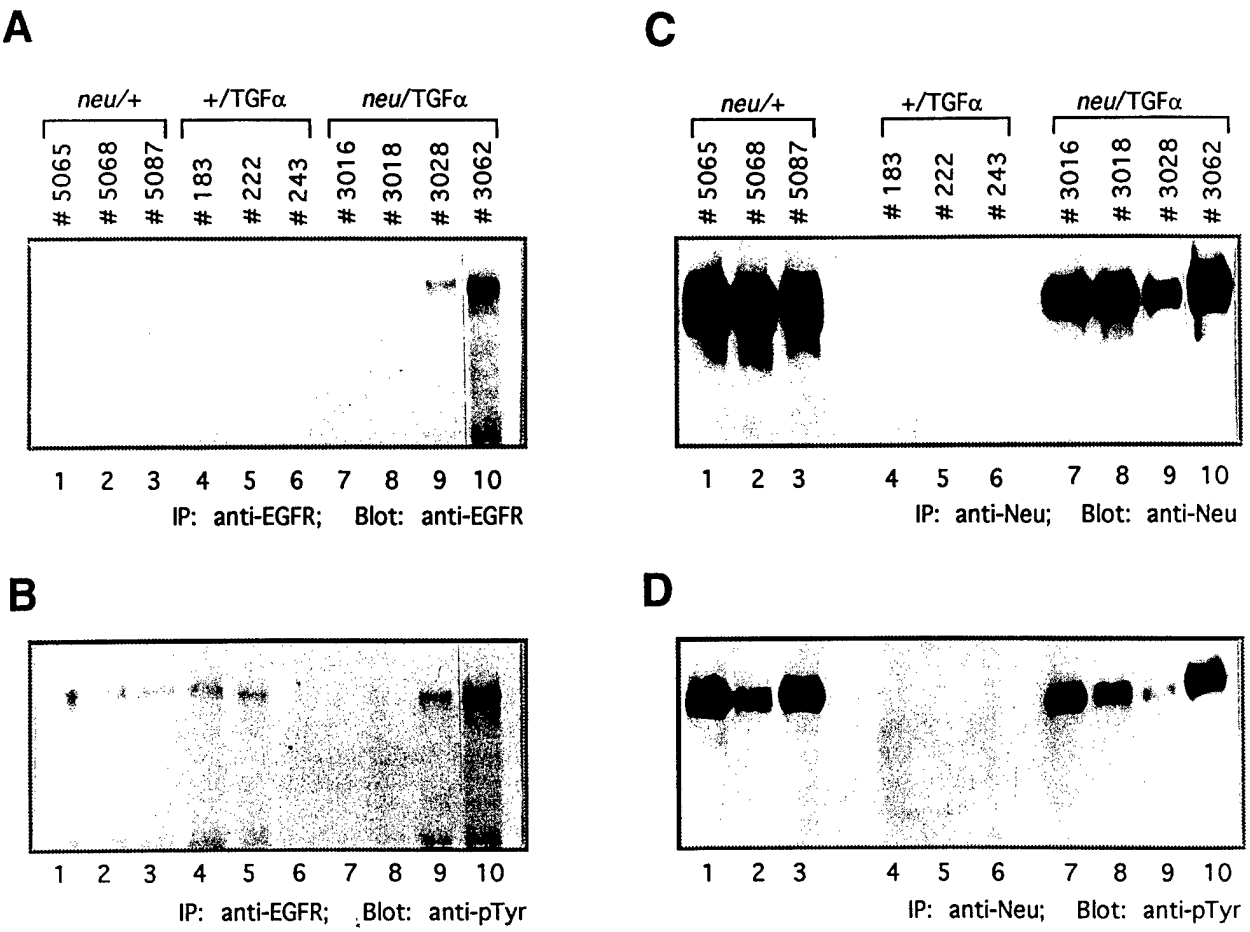

FIG. 7. Mammary tumor tissue from bigenic *neu*/TGF-α mice possess constitutively activated Neu. (A) Protein lysates from tumor tissue carrying either the MMTV/*neu* transgene (*neu*/+), the MMTV/TGF-α transgene (TGF-α/+), or both transgenes (*neu*/TGF-α) were immunoprecipitated (IP) with an anti-EGFR antibody and then subjected to immunoblot analysis with the same antiserum. (B) Tissue lysates identical to those in panel A were immunoprecipitated with anti-EGFR serum and subjected to immunoblot analyses with antiphosphotyrosine antibody 4G10. (C) Protein lysates identical to those in panel A were immunoprecipitated with the 7.16.4 monoclonal (anti-Neu) antibody and then subjected to immunoblot analysis with anti-Neu polyclonal antibody AB.3 (Oncogene Sciences). (D) Tissue lysates identical to those in panel A were immunoprecipitated with the 7.16.4 monoclonal (anti-Neu) antibody and subjected to immunoblot analysis with antiphosphotyrosine antibody 4G10.

the neoplastic mammary epithelium. The occurrence of inflammatory stroma in these tumors was due to the expression of TGF-α, since the MMTV/TGF-α mice also developed inflammatory stroma adjacent to the mammary epithelial hyperplasias. Because the MMTV promoter-enhancer is normally not active in the adjacent stromal tissue (21), the stromal hyperplasias observed in these animals were likely the consequence of local paracrine stimulation of the adjacent stromal cells by the adjacent TGF-α-expressing epithelial cells. Coexpression of TGF-α and *neu* in the mammary epithelia results in the epithelial dysplasias which frequently progressed to mammary adenocarcinomas (Fig. 4, 5, and 6). These observations strongly suggest that TGF-α and Neu can cooperate during mammary tumorigenesis in vivo.

The rapid induction of mammary tumors in the dual bigenic female mice correlates with elevated expression of both the TGF-α and *neu* transgenes. Interestingly, the mammary tumors induced by *neu* alone displayed evidence of altered transcripts (Fig. 1A). Indeed, previous studies have demonstrated that these altered transcripts encode mutant Neu proteins which possess constitutive tyrosine kinase activity (31). Consistent with these data, the levels of tyrosine-phosphorylated Neu in tumors derived from MMTV/*neu* mice were greatly elevated (Fig. 7D). In contrast to these observations, altered *neu* transcripts were not detected in mammary tumors coexpressing TGF-α and *neu* (Fig. 1A). Nonetheless, tyrosine phosphorylation of nonmutated Neu was detected in these tumor tissues, thus supporting EGFR-mediated transactivation of the *neu* proto-oncogene product (Fig. 7D). In addition to tyrosine-phosphorylated Neu, various levels of tyrosine-phosphorylated EGFR were also detected in mammary tumors from transgenic mice coexpressing TGF-α and *neu* (Fig. 7B). The reason for the highly variable EGFR levels in these tumors does not appear to be sampling error, since the same samples possessed elevated Neu. It is conceivable that the different ratios of the *neu* transgene to the endogenous EGFR may influence the phenotype exhibited by the tumors arising in these dual carriers. In this regard, it is interesting that the tumors arising in these bigenic mice exhibited a tubular or nodular phenotype (Fig. 6). However, determination of whether the ratio of the *neu* transgene to the endogenous EGFR influences these phenotypes awaits further analyses.

Unlike the tumors arising in parental MMTV/*neu* mice, which frequently possess activating mutations in the transgene

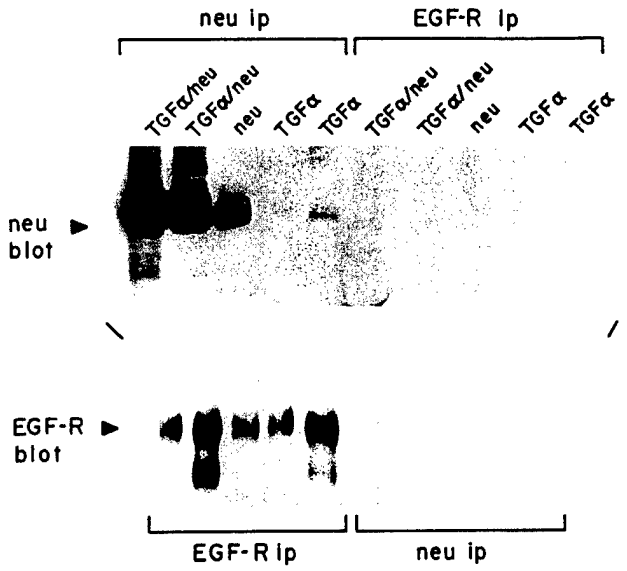

FIG. 8. Lack of detectable EGFR-Neu association in transgenic mammary tumors. Tumor membranes were prepared as described in Materials and Methods. Four hundred micrograms of membrane protein from each tumor was immunoprecipitated (ip) for 2 h with anti-Neu polyclonal antibody 21N or 986 anti-EGFR serum and Staph A cells. Precipitates were then subjected to immunoblot analysis with Neu (top panel)- or EGFR (bottom panel)-specific antiserum. Despite a detectable level of precipitable EGFRs in all of the tumors, EGFR was undetectable in all of the Neu immunoprecipitates.

(31), comparable *neu* mutations were not required for mammary tumorigenesis in bigenic *neu*/TGF-α mice (Fig. 1A, lanes 1 to 7). One possible explanation for the lack of activating *neu* mutations in these tumors is that Neu is activated through association with the EGFR. Consistent with this hypothesis, several groups have demonstrated in both fibroblasts and mammary epithelial cell lines that Neu can be transphosphorylated by the activated EGFR following EGF stimulation (1, 8, 13, 35). In fact, transphosphorylation of Neu can be mediated through the formation of a Neu-EGFR heterodimer (14). Moreover, EGFR-Neu heterodimers exhibit a 10-fold greater affinity for EGFR ligands (39). However, in tumors derived from *neu*/TGF-α mice, stable Neu-EGFR heterodimers were not detected (Fig. 8). Therefore, if heterodimerization between EGFR and Neu is involved in the synergistic induction of mammary tumors, the formation of these complexes is likely transient. This possibility cannot be ruled out by our experimental methods. On the other hand, these heterodimers have been reported only in cells with $>10^5$ EGF-binding sites per cell and after the addition of >10 nM exogenous EGF (8, 39). A lower level of EGFR in *neu*/TGF-α breast tumors may not allow adequate stoichiometric interactions between both RTKs and thus explain our inability to detect receptor heterodimerization. The ability of Neu to cooperate with the activated EGFR is consistent with a number of previous studies. For example, it has been demonstrated that EGFR and Neu can cooperate to transform cell lines in vitro (14). Conversely, it has been shown that administration of antibodies directed against either the EGFR or Neu reverses the transformed phenotype of cells coexpressing both Neu and the EGFR (38).

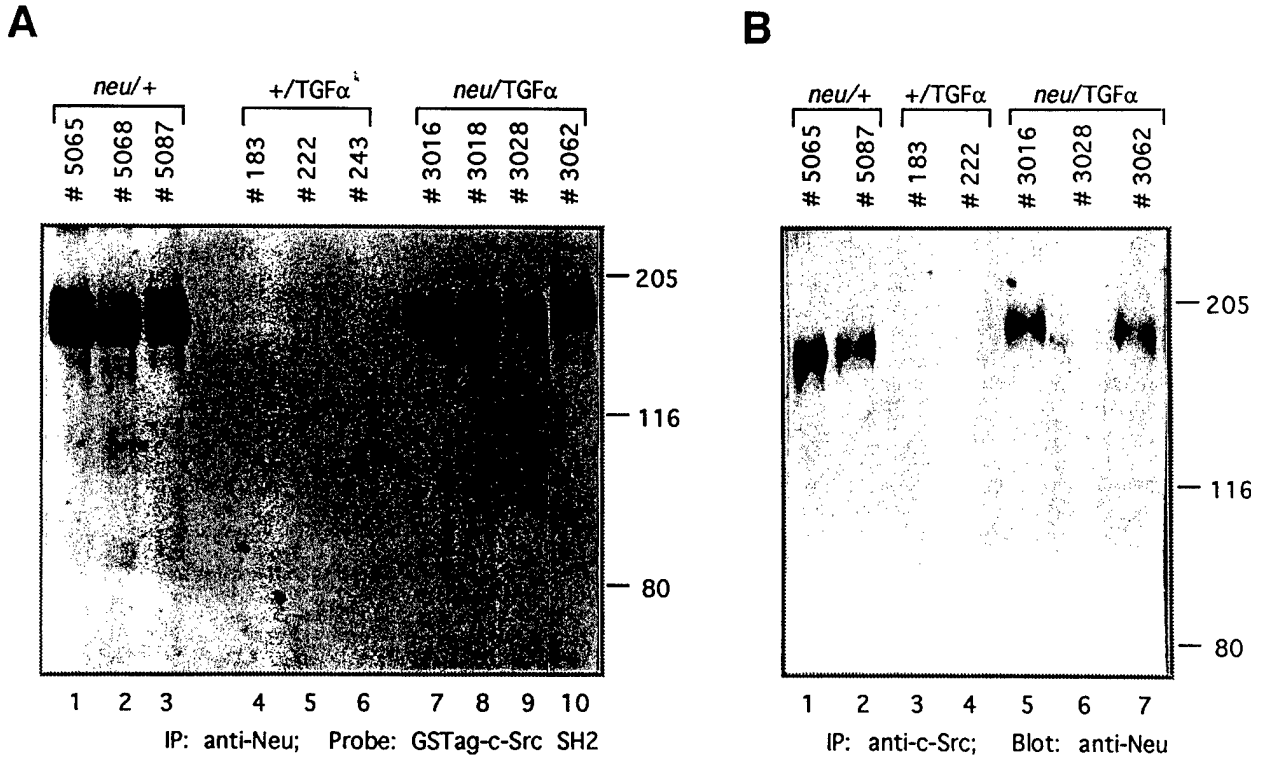

FIG. 9. c-Src is complexed with tyrosine-phosphorylated Neu in *neu*/TGF-α tumors in vitro (A) and in vivo (B). (A) Anti-Neu immunoprecipitates (Anti-Neu) from *neu*/+-, +/TGF-α-, and *neu*/TGF-α-expressing tumors were resolved in an SDS-polyacrylamide gel, blotted onto a polyvinylidene difluoride membrane, and probed with a radiolabeled GSTag–c-Src–SH2 fusion protein. (B) c-Src was immunoprecipitated (IP) (anti-c-Src) from the identical set of lysates and probed with anti-Neu serum.

Although it is clear from these results, as well as other observations, that activation of the Neu RTK by TGF-α results in synergistic transformation of mammary epithelial cells, the molecular basis for this cooperation is unclear. It is conceivable that activation of these closely related RTKs results in the recruitment of distinct but complementary signaling pathways to each of these receptors that then cooperate to transform the mammary epithelial cells. This hypothesis implies that each of these type 1 RTKs is coupled to distinct signaling pathways. In fact, several studies have suggested that coupling of the EGFR to the phosphatidylinositol 3′-kinase requires participation of the c-*erbB*-3 RTK (28, 34). We have demonstrated that the direct and specific interaction of c-Src with Neu is involved in signaling by the activated EGFR (23). Consistent with these earlier observations, we have shown that in mammary tumors induced by coexpression of *neu* and TGF-α, c-Src is complexed both in vitro and in vivo with tyrosine-phosphorylated Neu (Fig. 9). Although preliminary analyses suggested that c-Src activity was elevated in these *neu*- and TGF-α-coexpressing tumors, precise quantitation of the specific activity of c-Src in these tumors was problematic because of the extensive inflammatory stroma present in these tumors (Fig. 6).

Although these studies strongly suggest that TGF-α cooperates with Neu through the activated EGFR, it is unclear whether activation of EGFR is necessary for the induction of mammary tumors by Neu. However, several recent studies suggest that the activity of the EGFR is required for normal mammary epithelial proliferation. For example, a naturally occurring mouse mutant known as waved-2, which possesses a mutation in the EGFR catalytic domain that renders the EGFR functionally inactive (17), exhibits a severe lactation defect (7). Crosses between MMTV/*neu* transgenic mice and waved-2 mice should allow this question to be addressed.

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